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# PREFACE

A preface to this *Review* provides an opportunity to again express our thanks to those who have collaborated in the authorship of the present volume. Progress is so rapid in many of the fields of microbiology that reviewers are faced with difficult problems of selection and digestion of the pertinent literature. In addition, the selection of topics to cover the various aspects of general or specialized microbiology as they develop continues to be a major task. The Editorial Committee particularly welcomes suggestions and criticisms in this regard.

It is a pleasure, as in the past years, to extend our thanks to both our editorial assistants and other members of the office staff and to the George Banta Publishing Company for their painstaking care and cordial collaboration.

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# TOPICS AND AUTHORS

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## ERRATA

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pages 438, lines 3 and 4: *for* Leusen, Rhian & Stibbius *read* Lensen, Rhian & Stebbins

page 450, reference 17: *for* Leusen *read* Lensen; *for* Stibbius *read* Stebbins

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# ELECTRON MICROSCOPY OF MICROORGANISMS AND VIRUSES[1]

By James Hillier

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## Introduction

The application of the electron microscope[2] to the study of microorganisms and viruses had its beginnings slightly over a decade ago at the same time that the electron microscope became a reasonably practical instrument. The earliest work of Borries *et al.* (2), Piekarski & Ruska (3, 4, 5) Burton, Hillier & Prebus (6), and Kausche *et al.* (7, 8, 9) demonstrated that bacteria and viruses were not totally destroyed by the treatment to which they were subjected when examined in the electron microscope and that some details of structure considerably beyond the resolving limit of the light microscope could be seen. This was a very modest start which has been followed by a steadily increasing number of workers who have become interested in this use of the electron microscope and have published over 400 papers covering an extremely wide range of problems.

In view of the great diversity of the investigations that have been undertaken and the exploratory nature of many of them, a conventional review would undoubtedly degenerate into a documented index of the specimens that have been examined in the electron microscope with an unsupported estimate of the quality of the results. Since this has already been done in most of the general references on electron microscopy (1), it would appear to be more valuable to derive from the integrated literature an accurate definition of the present range of application of the electron microscope in microbiology and to indicate rather briefly those contributions which fall within this defined range.

## The Range of Application of Electron Microscopy

It is often stated that the electron microscope is a tool for demonstrating fine-scale morphology. While this statement is quite accurate, it cannot be left without considerable qualification. In the first place, here, even more than in light microscopy, the problem of artifacts is a major one which can never be dismissed from the research or from the interpretations.

The second qualification concerns the matter of resolving power and resolution. The practical resolving limit of the electron microscope is usually given as lying in the range 1 m$\mu$ to 2m$\mu$ (1, 10). While such resolving powers can be applied to most specimens encountered in the field of microbiology, the resolutions actually achieved are also dependent on the contrast in the

[1] This review covers approximately the period from 1940 to December, 1949.

[2] For a partial list of general references, see (1).

image and will vary widely within a single image. In fact, for the internal structure of well prepared, whole vegetative cells, the resolution achieved is rarely very much greater than would be obtained with a good ultraviolet light microscope. Thus, if there is structural differentiation in the image, it can be considered to exist subject, of course, to the possibility of its being an artifact. On the other hand, the absence of structural differentiation in the image does not necessarily mean the absence of structural differentiation in the specimen. The fact that even relatively large structures in the bacterial cytoplasm may not be demonstrable in the image has been given very little consideration in the literature. This is unfortunate as an awareness of this situation would have led to the development of methods for enhancing the contrast and thus making such structures visible.

Since the electron microscope provides information with regard to morphology, it is of value in any research problem involving questions of morphology with the provision that a suitable technique of specimen preparation is available or can be developed. This technique must take into consideration the fundamental requirements of the instrument, namely, the specimen must be desiccated and prepared in the form of a thin film less than 0.1 $\mu$ to 0.5 $\mu$ thick. On the other hand for a correctly stated research problem, the development of a satisfactory technique is often tantamount to solving the problem. With the foregoing points in mind, it is possible to derive from the literature a statement of the general types of research problems which are susceptible to solution by the methods of electron microscopy.

*Size, shape, and concentration of viruses and isolated bacterial structures.*—The simplest and most easily executed study is the measurement of the size, shape, and concentration of viruses or of isolated bacterial components. Obtaining such measurements is completely contingent on the ability of the worker to prepare sufficiently pure samples of the material or on his ability to recognize the significant structures. Recognition of a structure presupposes that a properly purified and identified structure has been examined previously. If the results obtained confirm those predicted from the indirect measurements that are implicit in many purification techniques, the electron microscope provides no more than a verification of the validity of the indirect method. On the other hand, the discrepancies that frequently arise are significant and should be the subject of further work. This type of research provides one of the few opportunities of detecting artifacts of preparation by independent measurements. This aspect of electron microscopy has been developed to the point where newly purified viruses are examined as a routine matter.

*The existence or nonexistence of characteristic structures.*—Studies which are also simple of interpretation but somewhat more difficult in execution involve the determination of the existence or nonexistence of characteristic structures. The appearance of such structures must have been previously defined by electron microscopic examination of purified specimens, by theoretical considerations based on indirect measurements, or by extrapolations from light microscope observations.

The realities of the cell membrane, cytoplasmic membrane, flagella, and nuclear apparatus which have been confirmed for bacteria are the major contributions in this category. Artifacts have not played an important role (except possibly to make the structures more visible) because of the rigidity and stability of most of the structures in question.

In the field of viruses, only a limited amount of work that fits this category has been done. This is mainly due to the fact that many viruses do not show characteristic morphologies in the electron microscope. Moreover, they are easily confused with other material that may exist in their environment. However, cell membranes and perhaps division-forms and nuclei have been established in the case of the rickettsia and pox group of viruses. An important exception is that of bacteriophage. Many of these have been shown to have a very characteristic morphology which, moreover, permits them to be easily identified in many environments provided they do not occur in close association with bacteria or other large masses of organic material.

*The delineation of fine-scale morphology.*—The delineation of the fine-scale morphology of microorganisms and viruses is a category in which the most important contributions of the electron microscope in microbiology will be made. However, until a serious and intelligent effort is made to solve the rather difficult problems of specimen preparation and artifact elimination, those contributions will not be forthcoming, and the full value of the electron microscope will not be realized.

As indicated in the above, the practical resolving power of the electron microscope lies below 2 m$\mu$, which brings molecules of molecular weights above approximately 20,000 within the range of visibility. Thus, a large fraction of the fine-scale organization of the bacterial cell or of the virus is potentially visible. On the other hand, as also indicated above, contrast limitations have restricted the achieved resolution within the bacterial cell to the range 80 m$\mu$ to 200 m$\mu$. In some viruses, the situation is somewhat better and resolutions of 20 m$\mu$ have been achieved. There are two contributing causes to this loss in resolution through lack of contrast (11). All of the structures involved are organic in nature with very similar densities which produce only slight differences in electronic scattering and hence only slight contrast. The second is really another manifestation of the same situation but comes from the superposition of many structures. For instance, in a hypothetical section of a bacterium cut at a thickness of 0.01 $\mu$, it is likely that structural differentiations at the macromolecular level could be resolved. However, in the whole bacterium, there are superimposed 100 such sections which mask the differences in scattering from the points of a single layer and introduce the utter confusion of so many superimposed structures. As would be expected from this theory, the structural differentiation that is possible in a virus is considerably better.

Contrast can be improved only slightly by changing the design of the instrument (11). Instead, it must come through the development of suitable specimen techniques. Several possibilities suggest themselves and are within reach of present day techniques. These are: (*a*) sectioning [Sections of 0.1

$\mu$ can be cut and could lead to a structural resolution of 25 m$\mu$ (12).], (*b*) selective staining [True selective staining seems feasible, but no serious attempts have been made to develop such a technique. This could also be combined with sectioning to give further improvement in resolution.], and (*c*) selective etching [This must involve a molecular etching of desiccated organisms whereby the products of the reactions are gases (13). It could also be combined with sectioning.].

The identification and elimination of artifacts is a problem which must be solved before it will be possible to provide an accurate delineation of the fine-scale morphology. This will be exceedingly difficult to do for at least three reasons: (*a*) There are no independent means of establishing hetereogeneous morphological details in the range of dimensions 0.1 $\mu$ to 0.001 $\mu$. (*b*) Morphology involves three dimensions, a fact which presents difficulties of recognition from different directions even if good representation in three dimensions is made possible. (*c*) Even at the 0.1 $\mu$ level, the morphology of a living organism is likely to be changing rapidly so that an almost infinite variety of instantaneous representations may be obtained, all of which may be accurate.

There can be no question but that the various writers are aware of the difficulties. Unfortunately, all too frequently the difficulties are discussed in the introduction to a paper and ignored in the interpretation of the results.

*The observation of morphological changes with time or as the result of interactions between different entities.*—Since it is assumed that all microorganisms and perhaps viruses are dynamic systems and since there must be a close relationship between the chemistry of the organisms and their morphologies, the electron microscope can play an important role by demonstrating the changes in morphology which occur normally or under the influence of specific agents. Obviously, research of this category cannot be undertaken until many of the problems of the preceding categories are solved. Moreover, a new problem is introduced if this category is not to be limited to gross changes. A single specific organism can be examined by means of an electron microscope at only one point in its normal development. This means that the direct study of the intricate interplay of fine structures will never be possible, for instance, in the way one can study by means of the phase contrast microscope, the behaviour of a living cell in tissue culture. This does not mean that one cannot study the appearance or disappearance, changes in dimensions, or even changes in arrangement of characteristic structures. However, such studies must be carried out statistically and under conditions where the artifacts are either eliminated or well defined.

Since these artifacts have been neither studied nor eliminated and since the fine-scale structure of the normal bacterial cell and the changes that occur in that structure have not yet been demonstrated, one is led to consider any research in this category as being very much premature. In spite of this there are many publications such as those on the multiplication of bacteriophages or on the morphological changes induced by antibiotics, which fall in this category.

## Techniques of Specimen Preparation

On surveying the literature, it is obvious that all too little serious consideration has been given to the problem of specimen preparation. This is probably due to the fact that the first technique suggested was quite adequate for the demonstration of viruses in purified preparation and of the more stable and larger bacterial structures. This phase of the work is now passing rapidly. Since it is now apparent that future advances in this field will be directly determined by the development of more satisfactory preparative techniques, some of these will be discussed critically but briefly in the following.

In the majority of preparative techniques used in the electron microscopy of microorganisms and viruses, a thin film is suspended across the opening or openings of a metal holder and constitutes the actual support for that part of the specimen which is examined. This film must be sufficiently strong and elastic to withstand considerable surface forces encountered, must be of minimum mass per unit area, and must be structureless to the limit of resolution. No film developed to date satisfies all of these requirements, but many almost do so. A number of organic materials such as collodion, "Formvar," and a number of plastics can be used to produce films of thickness between 10 and 20 m$\mu$ (1). All such films show a molecular roughness when shadow-cast (14), and the thinnest show a corresponding granularity by transmission (15). This roughness has minimum dimensions of 5 m$\mu$ and provides a major limitation on the smallest organic individual molecule that can be discerned (16). Attempts to produce smoother films by condensation *in vacuo* of light metal atoms on liquid surfaces have not been successful although extremely thin films of negligible scattering power have been produced (17). A great variety of methods of producing suitable films is now available (1). There are also a number of different types of films which have special properties that are desirable in some types of work (1, 17, 18, 19).

In addition to the mechanical problem of mounting the specimen, there is always the problem of obtaining sufficient contrast in the pertinent structures. This latter problem with the electron microscope has not yet been solved and very few, if any, attempts are being made to solve it. Of the two partial solutions offered in the literature, shadow-casting is most commonly used (16).

The many advocates of the application of the shadow-casting technique to biological problems consider it as a means of enhancing contrast. This is only a half-truth and a very misleading one. The shadow-casting can be said to enhance contrast only in that it emphasizes the surface contour of the prepared specimen at the expense of the internal structure. It would be more significant to consider shadow-casting as a method of replication since the information it provides is exactly the same as that provided by the other replica techniques used in electron microscopy.

Since shadow-casting technique is limited to the study of the outermost layer of a biological preparation, it is ideal for the measurement of highly purified viruses or for the identification of characteristic structures in the debris of destroyed cells. It is very misleading in any study of the internal

organization of viruses or bacteria in their natural state since by definition, internal structure cannot be made to manifest itself as a surface structure without disrupting that organization.

The second partial solution to the contrast problem is hardly applicable to problems of microbiology as yet. It involves the treatment of the specimen with compounds which contain heavy metal atoms and which have an affinity for organic material. Phosphotungstic and phosphomolybdic acids (20), osmium tetroxide, and calcium chloride are the only compounds that have been found useful in this respect. No real specificity for the internal structures of microorganisms has been shown by any of these compounds, except possibly osmium tetroxide. However, they can be useful for enhancing the contrast between some viruses and the supporting membrane (21).

The final general problem of specimen preparation is that of fixation. A careful study of the properties of different fixatives has not been made though some work has been started (22). Desiccation is the most common method of fixation, with formalin or osmic acid being used occasionally. The reason for this situation is that all organisms and some of the larger virus remain turgid and completely opaque to electrons when they are fixed by chemical means. On desiccation without fixation, most microorganisms flatten considerably (23). The actual amount of the flattening depends on the organism and its prior treatment. It has been possible to observe internal structures in only those organisms which flatten to less than 0.2 $\mu$. In a few isolated cases, extremely dense structures within thicker cells have been made visible by the use of higher voltage electrons (24).

*Specific mounting techniques.*—There are a number of variations of the technique of mounting bacteria or viruses from suspension. The simple evaporation of a drop of the suspension on a mounted film is the most direct. Such a drop may also be evaporated on a film-coated glass slide to be removed after further treatment such as shadowing or staining, or it may be evaporated on glass for the production of a preshadowed replica (16). For quantitative work with viruses, microdrops containing a sol of known concentration may be evaporated (25, 26, 27), or the viruses may be centrifuged directly on to a prepared membrane (28, 29).

In the case of the viruses, the suspension is usually the end product of the purification technique. Bacterial suspensions are prepared either by the direct suspension in distilled water or normal saline of the growth from a solid medium or by centrifugation and resuspension of the growth from a liquid medium. If normal saline is used, the salt crystals are removed after the preparation is dried by washing with distilled water. None of these techniques preserves any of the physical relationships of the original culture. Flagella are usually torn from the cells and then redeposited at random by the surface tension forces which occur in the last stages of drying. Similarly, any particles or bacteriophages which are present with the organisms are deposited on the film according to the whimsy of these surface tension effects. Placing biological significance on these drying patterns is a common error.

For many organisms, the use of distilled water followed by desiccation unquestionably introduces considerable distortions. The greatly increased concentration of salt, which occurs in the final stages of drying, can have an equally disastrous effect on the cells if saline is used. In spite of its many defects, this technique has been used for almost all of the bacteriological work with the electron microscope.

A pseudoreplica technique has been developed for thin surface colonies (23, 30). A dilute solution of a plastic is flowed over the colony, allowed to evaporate, and the resulting film floated off. If conditions are right, the outermost layer of the growth remains embedded in the plastic film and is thus removed intact. The method is particularly useful for studying the organization of a very young surface colony and the flagellation of the organisms. It has the disadvantages that the organisms are exposed to the organic solvent and that only the surface layer is held intact. The latter limitation is most serious in bacteriophage studies in which case viruses located elsewhere than on the surface of the growth are released and allowed to become absorbed or simply dried in a new location. While true replica techniques can be successfully applied to biological objects (1), their use in the study of bacteria has been limited to a few test cases (23).

A technique which comes closer to permitting the examination of organisms in their natural state involves growing them on a very thin collodion membrane which is in intimate contact with the nutrient medium (31, 32). If suitable wetting agents are present in the inoculum and if the moisture above the culture is controlled, young colonies can be examined with a minimum of physical disturbance. Desiccation artifacts seem to be minimized in this technique. On the other hand, it has the disadvantage that the macromolecular metabolic products of the cells are retained with the growth, reducing the contrast in images obtained. While the larger cells are held in place by this technique, the same cannot be assumed for macromolecular materials and bacteriophage. Thus, in preparations of this type, the physical organization of the fine scale extra-cellular components of the growth is not necessarily significant.

To overcome the problem of insufficient contrast for the internal structure of the bacterial cell, the sectioning technique of Pease & Baker shows considerable promise (12, 33). This technique should also be ideal for the study of bacteriophage multiplication. Unfortunately, no results beyond the original demonstration of the method have been published.

## The Bacteria

Excluding those which concern the interaction of bacteriophage with their hosts, there are in the literature approximately 125 papers which discuss the morphology of microorganisms as shown by the electron microscope. These papers cover almost as many different topics. Very few of the results have been checked by workers at other than the originating laboratory. Stimulating controversies have not arisen except on the problem of the significance of flagella. Very conspicuous by its absence is a critical experimental study

of the artifacts of preparation. The explanation for this rather unscientific situation lies in the fact that bacteriologists have had, in general, only limited access to electron microscopes and hence have tended to concentrate on specific problems that were of immediate interest. In spite of the unsoundness of this approach, several distinct contributions have been made. Almost exclusively, these involve proof of the existence or nonexistence of a definite structure.

*Bacterial cell wall.*—All types of bacteria studied with the possible exception of the spiral forms possess a cell wall (34 to 39). The cell wall is a very thin (less than 20 mμ thick), strong, pliable solid membrane. Since, in electron microscope preparations, the cell wall is rarely the outermost surface, shadow-casting has not revealed any significant fine structure. In the debris and occasional ghosts of phage-lysed cells, indications of a mosaic structure of elliptical segments have been found (32). It is not known if this structure has any significance relative to normal cells.

*Cytoplasmic membrane.*—Rod forms show convincing evidence of having a thin elastic membrane which surrounds the cytoplasm and which in normal cells is probably in contact with the inside of the cell wall (40). It is visible in cells from old cultures and in partly autolysed cells (41). High contrast methods can make it visible in healthy cells in which the contents of the cell have shrunk from the cell wall (42). The very symmetrical shrinkage which occurs in isolated normal cells indirectly indicates the existence of at least a surface layer with properties different than those of the cytoplasm. Such a membrane does not seem to have been demonstrated in the case of the cocci though the existing micrographs provide considerable indirect evidence that it is present. In the case of the spiral forms, there is no significant evidence.

*Capsules.*—Beyond a confirmation of light microscopic studies on capsules swollen by reaction with specific antiserum, the electron microscope has contributed nothing (43).

*Flagella.*—The reality of flagella was demonstrated immediately upon the application of the electron microscope to the study of bacteria (4, 44). They appeared as threads several microns long and approximately 20 mμ in diameter. The diameter varies somewhat among different species but is very constant for any one of them. Claims that the flagella of one species may have different diameters have been based on micrographs showing poor resolution and have not been confirmed. The flagella on most species are easily broken and easily detached from the cells. Furthermore, in the electron micrographs, a flagellum becomes invisible if it passes over or under a cell. These facts, taken in conjunction with the artifacts of preparation, make it necessary to disregard most of the early (and many of the recent) conclusions as to the origin and mode of attachment of flagella.

An exception can be made in the case of the vibrios where the single terminal flagellum is firmly attached. In this case, it passes through the cell membrane to a small polar cap or granule at the surface of the cytoplasm (45). By careful fixation *in situ* of partly autolysed cells of *Proteus vulgaris*, each

of the flagella of these organisms has been shown to terminate in a spherical granule located inside the cell membrane but outside of the cytoplasmic membrane (41). These results and those obtained by the pseudoreplica technique in which the flagella have the same appearance in immobilized cells (23) provide rather conclusive evidence that Pijper (46, 47) is in error when he proposes that the flagella are polysaccharide twirls which are the result of motility. On the other hand, the results can give no indication as to whether or not the flagella are responsible for motility. Attempts to demonstrate a periodicity in the structure of the flagella have been inconclusive except to indicate that if such a periodicity exists, it is at or below the 20 m$\mu$ level.

Some of the spiral forms, in particular *Treponema pallidum*, previously considered atrichous have been shown to possess flagella (48, 49). The artifacts of preparation have not permitted an accurate location or description of the points of attachment though there is some indication that a few flagella originate at each end of the organism.

*Cell nuclei and other cytoplasmic structures.*—Numerous intracellular structures have been demonstrated by means of the electron microscope. Unfortunately, owing to the great variety of conditions that can obtain, the numerous species involved, the many artifacts of preparation, and the almost complete absence of a careful study under controlled conditions, most of this work must be considered as very preliminary exploration with little general significance. In the case of the cell nucleus, the electron microscope has made a distinct, if limited, contribution. A few of the more consistent findings will be discussed here.

Cells from old cultures of nonsporulating rod forms show the presence of numerous granules in an otherwise clear cytoplasm (44). The largest of these are often 0.5 to 0.8 $\mu$ in diameter and located near the ends of the cells. The others are much smaller and vary greatly in size, location, and number. It has not yet been established whether these represent resting but viable cells or just dead autolysed remains. Cells from old cultures of sporulating rod forms show completely opaque spores, often still attached to very nearly empty cell membranes, and some cells similar to those found in nonspore formers.

Cells from young, actively growing cultures usually have a very dense cytoplasm which appears quite homogeneous in conventional electron micrographs. Using higher contrast methods (11, 15), some differentiation can be seen involving the existence of dense polar bodies and more transparent regions (32) that correspond with the location of the chromatin bodies demonstrated by cytological techniques using the light microscope (50). Unfortunately, in this case, the structures are defined with a resolution that is only a small factor better than is obtainable with the light microscope. Examination of the debris of cells freshly lysed by bacteriophage action, broken by ultrasonic treatment, or broken by chemical action gives indications that the intact cells may contain, in addition to one or more macromolecular

components, a number of discrete, organized, spherical structures with diameters in the 0.05 to 0.2 $\mu$ range. However, such a proposal must remain pure speculation until better techniques are developed (13, 32, 41).

For the present, the only contribution that the electron microscope can make to the study of the nucleus of the bacterial cell is the more accurate delineation of its structure. Moreover, this is possible only when the nucleus is identifiable in the electron micrographs on the basis of light microscopic and cytological studies. In sporulating cells of *Bacillus mycoides* which had been grown on a nitrogen-deficient medium, dense spherical granules, each with its own membrane and often in pairs connected by a less dense and apparently structureless material, were considered to be the nuclei (51). These conclusions were based on the identity of the structures observed by means of the electron microscope and those observed in living cells by means of the light microscope; special criteria of behavior in the living cells being the means of identification.

In rapidly growing cells, several workers have demonstrated by means of cytological techniques and the light microscope the existence of bodies which are regularly arranged in the cell and satisfy the cytochemical tests for desoxyribosenucleic acid [cf. (50, 52, 53)]. Electron micrographs of *Escherichia coli*, grown on a collodion membrane and given no treatment other than desiccation *in situ*, show less dense areas that correspond with the chromatinic bodies of the above-mentioned work (32). This experiment showed that the structural differentiation was at least not an artifact introduced by the chemical treatment. More recently, observations with the phase contrast microscope have revealed the same structures in living organisms and have established their connection with the nuclear structure by continuous observation of a dividing cell (54, 55).

An obvious, but not necessarily correct conclusion from the results on the study of nuclear structures is that there are similarities between the structure of bacteria and that of the cells of higher plants and animals. The structures demonstrated in the sporulating cells could be resting nuclei while those demonstrated in the actively growing cells could be primitive chromosomes in a continuous series of mitoses. The development of techniques for increasing resolution through increasing contrast and the development of cytological techniques for the electron microscope should enable that instrument to contribute considerably more to this problem.

*Spores, life cycles, and pleomorphism.*—In general, the spores of organisms are extremely dense and opaque to electrons of the energies normally used. Higher electron velocities reveal some structures, but contrast problems still constitute a serious limitation (56). Using the *in situ* growing techniques, the delicate empty spore-cases of *Bacillus megatherium* can be preserved, and some of its macromolecular structure has been observed (42). Similarly, the various steps in the formation and germination of spores have been observed (31, 56).

The electron microscope has been used to study the various constituents of a culture in which organisms are undergoing pleomorphic transformation

(57, 58). It cannot be used, however, to provide proof of the existence of a pleomorphic transformation. This would require the performance of an absolute experiment which is very unlikely with the electron microscope. An analogous situation exists with regard to the study of complicated life cycles. The electron microscope can indicate only the persistent appearance of characteristic forms. They must be identified by independent experiments. All of the publications related to these subjects must, for the present, be considered of questionable validity owing to the large scale artifacts introduced by the preparative techniques used.

## The Rickettsia

The electron microscopy of the rickettsia has been confined to a few scattered observations of their morphology (59 to 63). The preparative techniques have been such as to lead to maximum artifacts. However, it can be concluded that the rickettsia have structures similar to bacilli but that these are smaller and more variable.

## The Viruses

The electron microscopic study of viruses has in general followed a set pattern. First, specimens have been purified as much as existing techniques would allow and have been checked for biological activity. Second, the morphology of the suspected particles has been observed and checked against the data obtained by indirect means. Finally, if a very characteristic morphology was found, specimens were then made of material which was as near to the natural state of the virus as possible. Unfortunately, satisfactory sectioning techniques have not been generally available, so the crucial experiment of examining the virus within the infected cell has not yet been done.

*The psittacosis-lymphogranuloma group of viruses.*—The psittacosis-lymphogranuloma group of viruses has been investigated only scantily. The work published (64 to 68) indicates that these viruses are approximately spherical with diameters varying between 0.2 $\mu$ and 0.8$\mu$. This variation occurs even in the bodies of one species. They possess cell membranes and somewhat granular protoplasms. In all the pictures published, there are obvious distortions due to preparation and desiccation of the specimen. There is little if any evidence bearing on their mode of multiplication.

*Pox-type viruses.*—The elementary bodies of vaccinia (69 to 72), canarypox (71, 73), infectious myxoma of rabbits (71), ectromelia (71), and molluscum contagiosum (71) have been examined and found to have a surprisingly uniform morphology. In their dried state, they appear brick-shaped with rounded corners. While there are slight differences among species, their dimensions are also uniform—250 m$\mu$ to 300 m$\mu$ long, 170 m$\mu$ to 250 m$\mu$ wide, and 140 m$\mu$ thick. The difference between the thickness and width is undoubtedly partly due to drying on the membrane. These viruses show a characteristic internal structure consisting of a central dense body and four smaller and less dense granules situated at the four corners of the particle as seen. The fact that these structures are enclosed in a membrane can be

demonstrated by cytolyzing with sodium hydroxide (70). Peptic digestion of the virus of vaccinia removes the four smaller structures but leaves the membrane and the central particle (74). At least one group reports that desoxyribonuclease has some effect on the central granule (74). There is some suggestion that the viruses multiply by binary fission, final separation occuring at the corners. In some cases, crude material has been used, and aggregates suggestive of inclusion bodies have been shown.

The viruses of varicella, herpes zoster, and herpes simplex (75, 76, 77), which also belong in this group, do not have as distinctive morphologies, being more nearly spherical and somewhat smaller in size.

While various workers have considered the possibilities of using the slightly different morphologies and dimensions as a means of diagnosis, the results would appear premature in view of the discrepancies in the results obtained on any one species by different laboratories (76, 78, 79).

*Smaller animal viruses.*—A number of smaller animal viruses have been examined with the electron microscope; however, the contribution in these cases has been rather limited and consisted mainly of a verification of the shape and dimensions as inferred by ultracentrifugation and other biophysical methods. The group includes various strains of influenza virus (21, 80 to 83), encephalomyelitis virus (84, 85), rabbit papilloma virus (86, 87), mumps virus (87a), and Newcastle disease virus (88, 89). All of these appear to be spherical and rather dense, homogeneous particles. They have diameters in the range 20 m$\mu$ to 100 m$\mu$, and each species and strain has a rather narrow, but appreciable range of diameters. In the case of the Newcastle disease virus, filaments were found associated with the spherical particles if prepared in hypertonic concentrations of sodium chloride (90, 91, 92).

At least one human papilloma virus has been found to have such a uniform particle size that small cyrstals can be produced (93). A number of attempts to photograph the poliomyelitis virus have not given conclusive results (94 to 101).

More recently attempts have been made to advance the electron microscopy of animal viruses beyond the examination of purified specimens only. Elongated forms have been observed in preparations of influenza virus (102, 103). The significance of these has not yet been determined. Influenza virus particles adsorbed on red cell membranes of fowls have also :been examined (104, 105). Preliminary micrographs have been obtained of eastern equine encephalomyelitis virus in tissue culture cells (106).

Two independent methods of using the electron microscope to provide an absolute count of the number of particles per unit volume have been developed (25, 26, 28, 29). These should prove to be quite valuable for determining the molecular weight of the virus particles and the ratio of infective to noninfective particles.

*Insect viruses.*—Several insect viruses, particularly those of silkworm jaundice, have been studied by means of the electron microscope. The work has been concerned mainly with the isolation of the viruses and the demonstration of their morphologies (107 to 112).

*Plant viruses.*—A large number of different types of plant viruses have been examined to determine their sizes, shapes, and uniformity. In part, these include tobacco mosaic (7, 8, 113, 114), tomato mosaic (115, 116), cucumber mosaic (114), tomato-bushy stunt (114, 117, 118, 123), tobacco necrosis (114, 119, 120), southern bean mosaic (120 to 123), turnip yellow mosaic (124), squash mosaic (125), potato x-virus (126, 127), potato yellow dwarf (128), and tobacco leaf curl (129).

These viruses are characterized by their extremely uniform shape and size. Tobacco mosaic virus presents a possible exception in that the length of the rods varies according to the age and method of preparation (130). However, present evidence indicates that in the plant, the viruses have identical lengths (131, 132, 133) and that there is considerable doubt as to the infectivity of particles of other lengths found in old preparations (134). Electron micrographs of crude juice from infected tobacco leaves, smeared and dried but otherwise untreated, showed the tobacco mosaic virus particles arranged in bundles (135).

Small crystals of plant viruses, particularly of the smaller spherical forms, can be examined through the use of pseudoreplica techniques with the result that superb micrographs have been obtained of the lattice in the faces of these crystals (120).

The adsorption of specific antibodies by tobacco mosaic virus has been shown to produce an increase in thickness of the virus (136). In the case of small spherical viruses, (118) the antiserum appears to prevent crystallization [see also Grégoire p. 74 (1)].

*Bacterial viruses.*—Bacteriophages active against a large number of organisms have been examined with the electron microscope. Most, if not all, have very characteristic morphologies unlike any structures encountered in bacterial preparations. They are tadpole-like in that they have a dense head to which is attached a slender tail (137, 138). Depending on the strain and species, the head may be spherical, somewhat rectangular, or rod-shaped. The dimensions fall in the range 45 mμ to 100 mμ. The heads often show an internal differentiation. The tails range from 150 mμ long, flexible, and apparently single fibers to short (80 mμ) rods and bundles of fibers. A few strains of bacteriophages appear to be without tails. Whether or not this is a result of the method of preparation is not yet definite.

Purified bacteriophages of one strain have a remarkably uniform morphology (139), but in crude preparations, many abnormal forms can be found. When the organisms are grown and lysed *in situ* and examined immediately after lysis, many apparently incomplete forms of bacteriophage are seen (30, 140). In coliphages ($T_2$, $T_4$, and $T_6$), various treatments (ultrasonic and ultraviolet irradiations and some chemical treatments) will release the contents of the head leaving "ghosts" to which the tails are often still attached (137).

The very characteristic morphology makes the bacteriophages ideal subjects for electron microscope study. Unfortunately, limited contrast has not yet permitted the observation of the particles within the bacterial cell

before lysis. Examination of the preparation after lysis has provided some useful information regarding the existence of characteristic structures in the remains of the lysed bacterial cell (141). On the other hand, no existing meth- of preparation of cells after lysis can preserve, even approximately, the physical relationships among the bacteriophage particles, the macromolecular particles, and the remains of the bacterial cell. Diffusion of the components, the coagulating effect of fixatives, and finally, the gross rearrangement caused by surface tension during the final stage of drying serve to confuse completely the original arrangement even in *in situ* preparations. When the material is centrifuged from broth preparations, still another level of confusion is added. Furthermore, it must be emphasized once again, that the electron micrograph is a static representation which cannot indicate the direction of any change that is assumed to have been occurring. This direction must be determined by supplementary experiments. Thus, it would appear that little significance can be attached to the many published works which attempt to deduce the mode of multiplication of bacteriophage from micrographs of the products of lysis.

*Viruses and microorganisms related to neoplasms.*—Several publications (142 to 145) have appeared which purport to demonstrate the existence of characteristic virus-like particles in various neoplasms. For the present these can only be considered as highly speculative. In the sectioned material used, there are excessive artifacts which could be responsible for the results obtained. In any case, there is a complete absence at present of any criterion by which such particles could be identified. In the examination of "purified" materials, ordinary controls are of little value owing to the gross differences in the properties and physical structures of normal and malignant tissue. Finally, the all important, quantitative biological test of the infectivity of the purified material does not seem to have been made.

In at least one investigation (146) of purified preparations of the milk factor in suitable strains of mice, the biological test of infectivity has been made. Unfortunately, in this case, the electron micrographs were of poor technical quality and revealed no more than the uniformity of size that would be expected from the purification process.

Virus-like bodies which are either nonexistent or perhaps less numerous in normal cells have been observed in cultured rat sarcoma cells (147), in cultured epithelial cells from mammary carcinomas of mice of a milk factor strain (148), and in cultured chicken tumor cells (149). In the last case, the arrangement and structure of the bodies was sufficiently different from any of the normal cellular constituents to suggest that they represent the specific carcinogenic agent. However, in these cases, also, the matter is left in doubt by the absence of a biological test of the infectivity of highly purified preparations of these bodies.

Working on the old and often "disproved" hypothesis that neoplasms are the result of a bacterial infection, Wuerthele-Caspe and a group of collaborators have isolated successfully an acid-fast microorganism from several different animal and human tumors (150, 151). After several transfers either

in animals or in culture, the different isolates exhibited a surprisingly uniform morphology. They consist of dense spherical bodies $0.1\,\mu$ to $1.0\,\mu$ in diameter located in a matrix or coagulum of very small particles (10 $m\mu$ to 20 $m\mu$). Only the larger bodies could be seen in tissue by means of the light microscope and then only when acid-fast stains were used. Mice inoculated with these organisms developed a characteristic pathology including in particular the development of numerous apparently benign granulomas in the liver, lungs, heart, and kidneys. While this work has been well-controlled on conventional standards, the preliminary results indicate that more precise control must be devised before the results can be considered unequivocal.

## Conclusion

From the foregoing it can be seen that in spite of the many difficulties encountered in the study of microorganisms and viruses by means of the electron microscope, a very appreciable contribution has resulted from a rather superficial exploration of the field. The need for research of a much more fundamental type is obvious throughout. Fortunately, the specific problems which must be solved can be stated now with considerable accuracy. The analysis given here has been critical and even skeptical in an effort to emphasize the pitfalls and to point out the areas in which profitable research can be undertaken at the present time. Seen with the clearer perspective resulting from a decade of exploration, the potential value of the electron microscope in the field of microbiology appears to have been increased considerably.

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# BACTERIOPHAGES[1]

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In this review we shall attempt to bring the readers up to date in the rapidly expanding field of research on bacterial viruses. For earlier work the reader is referred to Fong's paper in this Review last year (1), or to the general reviews of Delbrück (2) or Anderson (3, 4,). We shall discuss first the physical and chemical properties of the phage particles themselves and then turn to their dynamic properties: their adsorption on host cells and their multiplication within and liberation from the cell. We shall discuss how these viruses mutate and exchange characters within the cell.

## Morphology

The electron microscope has been of the greatest use in determining the sizes and rather complicated shapes of the bacterial viruses and in the past year three books describing its use have appeared (5, 6, 7). A method (8) of spraying suspensions of virus in microdroplets onto the specimen screen now makes it possible to determine the concentration of total (active plus inactive) virus particles in suspending media containing volatile salts such as ammonium acetate. A method of reducing artifacts due to surface tension has been developed (9).

A phage attacking *Streptococcus lactis* has been found (10) to have a head 70 m$\mu$ in diameter and a tail about 150 m$\mu$ long and 30 m$\mu$ in diameter. Some beautifully clear micrographs (11) of purified phage T7 have shown it to be essentially spherical with a diameter of 51 m$\mu$, but with a bilobar arrangement of internal structure. It seems odd that electron micrographs of shadowed T7 particles show them to be as large as 73 m$\mu$ in diameter unless there is some creep of the shadowed metal on the surface of the particles, or unless the virus but not the shadowing gold shrank under electron bombardment.

Early micrographs of the even-numbered viruses in the T set had suggested that they had membranes surrounding the structures within the heads. It now appears that these head membranes have osmotic properties (12) for when T2, say, is equilibrated with salts or other solutes at concentrations greater than 2 M and then suddenly placed in dilute solutions the virus is inactivated. Electron micrographs of T2 treated in this manner have shown "ghosts" of the phage particles—empty head membranes with tails attached. When the solute is diluted slowly, however, the phage particles

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appear normal and retain their activity. It would seem that the head membranes are opened up during osmotic shock and allow their contents to escape into the medium.

The thermal inactivation of the T phages has been reported by Adams (13) to follow first order kinetics. In general these phages are much more rapidly inactivated in 0.1 normal sodium chloride than in broth. The addition of $10^{-3}$ M concentrations of such divalent cations as $Ca^{++}$, $Mg^{++}$, $Ba^{++}$, $Mn^{++}$, or $Cu^{++}$ markedly reduces the rate of inactivation of T5, which is of special interest since this virus specifically requires $Ca^{++}$for its multiplication within host cells (14). It would be interesting to know whether it is such cations as these or organic materials which stabilize the phages in broth.

Foster, Johnson & Miller (15) have studied the influence of hydrostatic pressures on thermal inactivation of phages in broth containing 0.5 per cent sodium chloride. At atmospheric pressure T1, T2, and T5 were inactivated at the same rate at 66°C. as T7 was at 60°C. Pressures up to 10,000 pounds per square inch retarded the thermal destruction of T1, T2, and T5 but accelerated that of T7. The rates of inactivation were increased by 0.005 *M* added phosphate, but slowed down by added 0.005 magnesium ion in all but T7, whose rate was unaffected. It would appear that divalent ions may stabilize viruses by bridging some of the weaker structures in their makeup which disintegrate thermally. It may be interesting in this connection that formol has been reported (16) to increase the thermal stability of a phage antigen.

Some phages seem to be quite resistant to the action of bactericidal agents and can be concentrated and purified by alcohol precipitation (17) and stored under thymol (18) to prevent bacterial contamination.

## Chemical Composition

Kerby *et al.* (11) have made a careful study of the composition of T7 phage purified by differential centrifugation. The material they analyzed appeared to contain $10^{-15.4}$ gm. of protein for each plaque-forming unit, which would be the weight of a sphere some 85 m$\mu$ in diameter. This is in fair agreement with the diameter of 73 m$\mu$ seen for shadowed T7 particles in the electron microscope. Nucleic acid, based on the phosphorous content, accounted for 38 per cent of the weight of the preparation which is close to the value found for the unrelated phage T2. Near neutrality the material in concentrations from 4.7 to 0.25 mg. per ml. had a sedimentation rate of 470 S, but when diluted to 0.021 mg. per ml. the rate was only 325 S. At pH's below 5 two fractions (381 S and 35S) were observed, while above pH 10 two other fractions (464 S and 19 S) were found, as though the extremes of pH had caused fragments to split from the particles. On the other hand the pH optimum for stability of infectivity lay in the more narrow range 6 to 8.

Putnam, Kozloff & Neil (19, 20) have undertaken a similar analytical task with T6 and obtained for it two sedimentation boundaries (790 S and 1030 S) as though relatively stable virus clumps were present. In electrophoresis studies T6 moves toward the anode between pH 5.1 and 7.6.

In 1948 Polson (21) reported diffusion constants for T3 and T4 activity that were in fair agreement with their particle sizes as determined in the electron microscope, but a report of later studies of T3 at lower virus concentrations (22) gives values some 20 times as large. In view of the extreme care with which rates of diffusion must be measured (23), their suggestion that a virus particle may have motility should be backed by very strong evidence before being taken very seriously.

### Action of Radiation

The target theory (24) of the action of ionizing radiations is designed to give one an estimate of the volume of relatively sensitive material in biological systems which is responsible for their continued activity. Pollard & Forro (25) have tested this theory by deuteron bombardment of dried T1 and find that it presents the volume of a $28 \pm 4$ m$\mu$ sphere. This is in fair agreement with the earlier value of 36 m$\mu$ obtained by x-irradiation (26) but both values are smaller than those observed with the phage particle as seen in the electron microscope. In working with deuterons, corrections are made for the inactivating radiation of a deuteron as it passes by the target, while with x-rays highly reactive intermediates produced outside the target must be inactivated before they can react with the sensitive biological system (27).

Although thousands of quanta in the ultraviolet must be absorbed for inactivation, many self-reproducing biological systems appear to be inactivated in an exponential fashion and with an action spectrum which roughly resembles the absorption spectra of the nucleic acids (28). But Kelner (29) has discovered that a large proportion of irradiated fungus spores and bacteria can be revived by subsequent illumination with visible light as though a light-sensitive poison had been produced by the ultraviolet (30). Such illumination of the T phages themselves subsequent to UV irradiation fails to reactivate them, but as we shall discuss later, Dulbecco (31) has found that a large proportion can be reactivated by illumination in the violet region after the particles have been adsorbed on their host cells.

### Interaction of Phages with Host Cells

*Typing of bacteria.*—Soon after their discovery it was realized that the specific lytic action of the bacteriophages could be used in the identification of bacterial strains found in nature. Indeed, this method is frequently more sensitive than the classical methods of immunology. Strains of *Staphylococcus aureus* (32 to 36), *Xanthomonas pruni* (37), *Bacillus polymyxa* (38), typhoid, and paratyphoid B (39, 40) have been typed recently by the phage technique. The relative frequency of 24 *Salmonella* types has been found to differ in the various regions of France (40) by the use of this technique. In some cases typing has been rendered difficult by the presence of phages contaminating the cultures to be tested, but Dunbar (41) has succeeded in rendering typable phage-contaminated cultures of *Salmonella typhosa* by growing them in the presence of antiphage sera. The phenomena customarily employed in

typing (presence or absence of lysis or of plaque formation) probably involve the whole gamut of relations between phage and host cells from adsorption, multiplication, lysis, interference, lysogenesis, and mutation of cells to resistance to virus activity. The data do not tell which of these may be responsible for the apparent resistance of a particular strain to a given phage. It is only after rather extensive studies like those with the T set of phages that the responsible mechanisms can be worked out. It is to this type of study that we now turn.

*Adsorption.*—In many ways the primary reaction between viruses and host cells is more amenable to direct study than the subsequent developmental processes of the resulting virus-host complex. And since adsorption is quite specific, a key to the mechanism of virus activity must lie in the reaction. Certain aspects of adsorption of the T phages have been discussed in recent reviews (42, 43). In the case of T4 we have a few experimental tools for the study of its adsoprtion. Certain strains of T4 are not adsorbed on host cells unless the virus particles have been activated by an amino acid cofactor such as L-tryptophane (D-tryptophane is inactive). At least six cofactor molecules seem to be required for adsorption, but activity is lost at a first order rate when cofactor is removed, as though the loss of only one molecule resulted in complete loss of adsorbability. Once activated, the rate of adsorption is almost equal to the rate of collision in quiet media, but in violently agitated media adsorption does not occur. It would appear that adsorption of T4 may be a dynamic process of degradation and/or synthesis which requires a time of the order of seconds before the virus is adsorbed on the cell in the sense that it is committed to further activity on that particular cell.

Like the development of the complex, adsorption of a virus is a cooperative affair between virus and host. Further evidence that the host's polysaccharides are involved has been obtained by Beumer (44). In the case of the T phages Miller and Goebel (45) find that the polysaccharide is combined with a lipoprotein. T2 and T6 are active on both wild type (Phase I) of *Shigella sonnei* and its mutant (Phase II) while T3, T4, and T7 attack only Phase II. Now the characteristic antigens of Phase I and Phase II have both been purified and found to be lipocarbohydrate-protein complexes which are serologically distinct. Lysis of neither Phase I nor Phase II bacilli by T2 or T6 is inhibited by the homologous type-specific antigen. In the presence of appropriate cofactors, however, lysis of Phase II bacilli by T2, T4, and T7 is specifically inhibited by Phase II antigen but not by Phase I antigen. The carbohydrate residue obtained from Phase II antigen after degradation by proteolytic enzymes can also inactivate T3, T4, and T7. The authors conclude that T2 and T6 combine with a different class of antigens than T3, T4, and T7, although the results do not exclude the possibility that T2 and T6 might be able to destroy Phase I and Phase II antigens or that the combinations that these viruses form with them may be weak.

Beumer-Jochmans (46) has observed that adsorption of phage on nonpathogenic strains of staphylococci is unaffected by normal sera but, as is well known, such adsorption is inhibited by specific homologous anti-

bacterial sera. In the case of pathogenic strains of staphylococci she has reported that normal sera from many mammals inhibit lysis by phage. The activity resides in the pseudoglobulin fraction of the normal sera and is not due to a precipitin. Since the serum fraction inhibits the inactivation of the phages by extracts from phage-sensitive strains of bacteria, it is thought that it may interfere with one or more of the steps involved in the adsorption process of phage on sensitive pathogenic strains.

The adsorbability of the staphylococcus phages studied by Rountree (47) seems to be correlated with the coagulase production of the host cells. Two classes of phages were studied: (*a*) those which lysed coagulase-positive cultures of human or animal origin, and (*b*) those which lysed coagulase-negative cultures of human origin. The phages of class (*a*) were inactivated and presumably adsorbed on heat-killed coagulase-positive cultures whether they were able to lyse the intact cells from such cultures or not. On the other hand, there were no observable reactions between coagulase-positive and coagulase-negative cultures and cells. Viscid mutants of a number of strains were resistant to lysis by phages, but still adsorbed them. Since most of the staphylococci studied here were lysogenic, it is not clear whether the absence of lysis by adsorbed phage implies interference with an intracellular phage or the induction of lysogenicity in the putative host for the newly adsorbed phage (34).

*Intracellular growth.*—Adsorption of the T phages rather quickly transforms both the host cell and the virus into a new entity which may be termed a virus-host complex. It has not been possible to recover the infecting virus particle from the newly-formed virus-host complex, either by premature lysis of the cell or by sonic disintegration (48). It would seem that on infecting a cell the virus has changed its state (49). As development of the complex proceeds, a few daughter particles begin to make their appearance only after about 2/3 of the latent period has passed. The number of the daughter particles then increases rapidly until the end of the latent period when the cells begin to lyse of their own accord (48).

The host cell is altered, too, as cytological studies show. Within 5 min. after the adsorption of five out of six phages studied by Quersin (50), the characteristic chromatic granules of the host (Shiga) are seen to be altered in a manner characteristic of the adsorbed phage. Such agents as chloroform, lysozyme, or alexin did not alter the nuclear granules in this manner. Characteristic alterations have been noted by other authors working with *Pasturella pestis* and *Escherichia coli* phages (51, 52, 53). With the T phages, for example (54), the relative rate of disruption of the granules of *E. coli* B is paralleled by the relative "interfering power" of the phages, T2 acting faster both in disruption and in competition than T7, and T7 in turn being faster in both respects than T1. With T2 the disruption of chromatin at 5 min. is followed by swelling of the cell and its filling at 15 min. with granular chromatinic material, probably the daughter virus particles. Single infection with ultraviolet inactivated T2 simply causes the host's chromatin to fade away, although the intensity of ultraviolet adsorption of the cell increases.

With T7 the chromatin converges into one large mass before lysis, a phenomenon not induced by irradiated T7. The other phages in the T set, even when inactivated, caused an accumulation of chromatin, although no active phage could be recovered from the cells even by lysing them artificially. It is to be hoped that the electron microscope will show further details in the above intracellular mechanisms. Some beautiful micrographs of bacteria undergoing phage action have been published recently (55, 56, 57), but as yet it is impossible to relate them to the known life cycles of the phages studied, and indeed some of the inferences reported have been at variance with almost all that is known about the physiology of phage multiplication.

*Growth requirements.*—We shall deal mainly with the literature which has appeared since Cohen's recent review (58) in which the effects of nutrients and metabolic inhibitors on development of virus-host complexes is well covered. Certain acridines have been found (59) to affect the time and extent of gross lysis of a number of organisms and these effects can be neutralized by ribonucleic acid, riboflavin, thiamine, nicotinamide, and sodium lauryl sulfate, although the details have not been studied. Five hundred substances have been tested for inhibitory effects on the reaction between a phage and *Pseudomonas pyocyanea* (60) but none inhibited phage at concentrations which would permit growth of the host. Thymol does not hinder phage adsorption or lysis of host cells sensitive to certain phages studied by Wahl & Blum-Emerique (18), but it does hinder their multiplication. The authors take this as evidence for independence of phage multiplication and lysis. The inhibition of T2-host complexes by desoxypyridoxine is reversed, not only by pyridoxine but also by fatty acids, glucose-6-phosphate, pyruvic acid, and somewhat less by lactic, malic, fumaric, or succinic acids (61). While streptomycin quickly blocks growth of staphylococci it only delays lysis of the organism by a phage and allows a residual turbidity to remain as reported by Faquet & Edlinger (62, 63). In none of these latter studies is it possible to tell what events in the series of reactions between virus and host are blocked by the metabolic inhibitors.

Calcium is required for the multiplication of certain phages, namely S13 (64), T1 (65), and T5 (14). When T1 (65) is incubated in water and then added to B in broth, it loses its ability to form plaques at a rate equal to the rate of adsorption of normal T1. The "sensitization" by water can be prevented by the addition of $10^{-4}$ *M* calcium chloride. Also, after "resensitization" by incubation in water, T1 can recover its ability to form plaques during incubation in broth (which contains calcium). It would thus appear that an adsorbed T1 particle must carry with it calcium, or possibly some other substance bound to it with calcium, if it is not to be irreversibly inactivated on adsorption.

With T5 (14) the situation is somewhat different. T5 particles are readily adsorbed on host cells in the absence of calcium, and prevent them from multiplying. But the virus-host complex does not develop until $10^{-4}$ *M* calcium ion is added. Forty minutes after the addition of calcium, phage is liberated from a fraction of the complexes which decreases the longer they are left

without calcium. Since the normal latent period is 40 min. it appears that an early stage in the development of T5-host complexes is interrupted by a lack of calcium and that the addition of calcium gives it a chance to proceed to the liberation of daughter particles.

It now appears that many virus particles which have been irradiated with ultraviolet light contain the elements for establishing a similar block in the development of the complexes they form with their host cells (31). Virus particles are damaged by ultraviolet irradiation (λ2537) in such a way that the logarithm of the number of surviving particles as measured by their ability to form plaques in the dark is very nearly a linear function of the dose of irradiation. However, the number of survivors is much greater if their ability to form plaques is measured in the light. As the light intensity is increased, the proportion of reactivated phages reaches a limit, as though the ultraviolet-irradiation had produced two kinds of damage: photoreactivable and nonphotoreactivable. These two kinds of damage appear to occur with comparable cross sections which differ, however, from phage to phage.

Dulbecco (66) has studied this effect in some detail and finds that the reactivation is accomplished by illumination of the virus-host complex, but not by illumination of the virus alone or of the host cells just before addition of irradiated virus. The reactivation has an action spectrum with a maximum at λ3650. This photoreactivation can occur a few seconds after addition of irradiated phage to sensitive bacteria, but only under conditions favorable for the adsorption of phage on host cells. It would thus appear that ultraviolet-irradiation produces aberrations in the phage which block an early stage in the development of the virus-host complex. While in this state, however, the block to development can be removed by illumination with visible light.

In quantitative studies (66) it is found that at low intensities of illumination the probability of photoreactivation occurring in a virus-host complex is proportional to the dose of illumination, but a maximum rate is reached as the intensity is increased. Photoreactivation also depends on temperature in a complex way, but the rate appears to be independent of the dose of ultraviolet used for inactivation. To explain these observations a rather complex theory is proposed: irradiation of a phage particle produces one or more inhibitor molecules, only one of which is capable of blocking an early step in the development of the complex which the phage particle might form with a host cell. On or within the cell is a receptor which is capable of destroying the inhibitor if it should become detached from the structures whose activity it is inhibiting. The inhibitor becomes detached only when some pigment associated with the system absorbs a light quantum. Then, if the receptor does not inactivate it, the inhibitor returns to the essential site again until another quantum ejects it. The rate of reactivation thus depends upon the fraction of the time the inhibitor is available to the action of the receptor. The rate is thus proportional to the light intensity for low intensities. For high intensities the inhibitor is off its site most of the time, so that the rate of photoreactivation reaches a limit determined by the avidity of

the receptor. The avidity of the receptor, in turn, is considered to be a function of the temperature and physiological state of the bacterium. The theory seems to account for the known facts of photoreactivation. Although its details may not stand the test of time, it is of particular value in the present discussion in pointing out the types of complexity to be anticipated in the development of a relatively simple biological system.

*Lysis.*—Very little is yet known about the final process of lysis of the virus-host complex or of the nature of the materials present in the lysate. In the latter connection, the lysis of a resistant strain of *Streptococcus lactis* (Leg 5) in a mixed culture with a sensitive strain (Strain 19) and its bacteriophage (Strain 5L) has been reported (67), but the mechanism is still obscure; similar observations have been made with staphylococci (68). The presence of an enzyme which decapsulates both living and killed cells of *Klebsiella pneumoniae* has been reported to be present in a phage lysate (69).

According to Maurer & Woolley (70), the lysis of *E. coli* B infected with T2 can be inhibited by 1 per cent citrus pectin and to a lesser extent by other pectins added to the medium before mixing virus and host cells. This phenomenon permits an experimental approach to the problem of lysis and may be related to the inhibition of lysis observed when the $r^+$ strains of the T-even phages are adsorbed on complexes formed by $r^+$ strains and their host cells. The phenomenon has also some features in common with the phenomenon of lysogenicity to which we now turn.

Some bacterial cultures seem to carry bacteriophages with them which fail to lyse an appreciable proportion of the cells in the culture but produce lysis and plaques when allowed to act on other sensitive or "indicator" cultures (35). Many lysogenic organisms have been found to retain their phages through mutations to resistance to an antibiotic such as streptomycin (71).

Rountree has recently made considerable progress in the study of lysogenicity in the staphylococci and has published an excellent review (72) of her work. The phenomenon is of wide occurrence in these organisms, for she finds that 27 out of 30 strains studied are lysogenic (73), although there may not have been suitable indicator strains for the phages contained in the remaining three. Neither serial isolation of colonies nor growth in antiphage sera or in citrate, to which certain of the phages are sensitive, has changed the lysogenicity of any of the 27 strains over a period of from one to five years. It would thus appear that every cell in these cultures is infected and passes the phages to its offspring during division. A small amount of free phage has been found in filtrates of young cultures of 25 of the strains studied, suggesting that a small proportion of the cells liberate phage. Of seven cultures chosen for more intensive study, one produced three different, but serologically related phages, while another produced five different phages belonging to three different serological groups (74). Evidently in these systems the host can support its own growth while harboring many strains of virus.

## Genetics

*Mutations of host cells to resistance.*—Lea & Coulson (75) have given a detailed statistical analysis of the distribution of mutants in growing cultures which should be useful in determining the rates of mutation of bacterial cells to resistance to a phage. Latarjet (76) finds that neither the "strong" carcinogen methylcholanthrene nor its photo-oxide increases the rate of mutation of B to resistance to T1, while "weaker" agents such as desoxycholic acid induces numerous mutations. In later work a water soluble series of agents showed a strict parallelism between carcinogenicity and mutagenicity (77). In a remarkable heterozygous diploid strain of *E. coli* K-12, Lederberg (78) finds that a factor determining sensitivity to T1 is dominant to the allele for resistance. Evenutally we may look for genetic relations between factors determining sensitivities to the various phages and perhaps to nutritional requirements, too.

*Mutations of bacteriophages.*—Recently there have been two attempts to induce mutations in phages by external agents. Using nitrogen mustard, Silvestri (79) has treated T2 phage, adsorbed the treated phage on B, and plated the virus-host complexes before they burst on the indicator strain B/2. He finds an apparent increase in frequency of appearance of the mutant phage T2*h* ("*h*" to indicate an activity on a new host) active on B/2, which parallels the killing effect of the nitrogen mustard. From his results he cannot tell whether the agent had induced the phenotypic character directly or whether, as seems more probable, it was really the daughters of the treated particles which showed the *h* character. The possibility of selection seems not to have been ruled out either, since the sensitivities to nitrogen mustard of T2 and T2*h* do not appear to have been compared (82).

Latarjet (80) has studied the induction of T2*h* mutants by ultraviolet irradiation. T2 and T2*h* are equally sensitive to the radiation used. Direct irradiation of T2 simply produced lethal effects, as will be discussed later. However, by irradiating T2-host complexes at various times during their development, 10 to 15-fold increases in the ratios of T2*h* yield to that of T2 were obtained from the survivors, provided the irradiation was given 7 min. or longer after their formation. It was at this time after infection that x-ray inactivation curves began to assume a multiple-hit character, showing that true multiplication of phage-forming units had begun within the developing complex (81). The proportionate increase in the number of mutants after irradiation still may have been due to selection, however, since irradiation may leave as survivors those virus-host complexes which contain the greatest number of nascent phage particles, hence also those in which spontaneous mutations are most likely to have occurred (82).

*Genetic recombinations between phages.*—As is now well known, the simultaneous infection of a cell of *E. coli* B with more than one type of T-even phage yields a virus-host complex which is often capable of producing not only the infecting strains of T-even, but hybrids between them as well. An excellent review of phage genetics by Delbrück has recently appeared

(83), and the reader is referred to the papers of Hershey & Rotman for further details (84, 85).

Dulbecco (86) has estimated the number, $K$, of T2 particles which can contribute their character to the yields from individual virus-host complexes. Assuming that $m$ T2 particles and $n$ T2$r$ particles are adsorbed on a bacterium, but only $K$ of them can "multiply," Dulbecco has calculated the chances that none of the particles of one of the infecting types be included in the multiplying group. This would be the probability that the yield would be unmixed. Experimentally he finds that mixed multiple infection with T2 and T2$r$ does indeed yield more complexes which produce particles of one type only than would be expected if there were no limitations to the number that can multiply. The results are in fair agreement with theory for a limitation of the number to eight or ten.

Luria & Dulbecco (87) have reported the details of their work on the mutual reactivation of ultraviolet irradiated phages infecting the same host cell. They found that T2, T4, T5, and T6 particles inactivated by ultraviolet light, at a logarithmic rate, are still adsorbed on sensitive bacteria. Bacteria infected by only one phage particle are not lysed and do not yield phages, while a certain fraction of the bacteria infected by more than one such particle is lysed and yields active phage. This fraction augments with increasing numbers of adsorbed phage particles, and decreases as the dose of irradiation is increased. Since irradiated T-even phages can "mutually reactivate" each other, but not irradiated T5, the assumption is made that the phenomenon is caused by genetic recombination between phages. It is postulated that irradiation produces lethal mutations in a number of genetic "units" of a phage particle and that genetic recombinations between phage results in the production of active daughter particles in those complexes which contain a complete set of active genetic units. Statistical analyses of the results to be expected from this mechanism fit the data obtained in the dark, if it be assumed that T2 contains 25, T4 contains 15, and T6 contains 30 such genetic units. It will be interesting to see how mutual reactivation is affected by photoreactivation of the virus-host complexes, for as previously mentioned ultraviolet-irradiated phages appear to undergo two kinds of damage, one of which can be largely eliminated by illumination of the complexes they form with host cells.

## A Perspective

The year 1949 saw the passing of Professor Félix d'Hérelle whose independent discovery in 1917 of "un microbe invisible, antagoniste des bacilles dysentériques" aroused the interest of the scientific world. It is d'Herelle's early interest in the nature of bacteriophages that has attracted physicists, chemists, and biologists to the field. For they see in this material a chance to unravel the primary mystery of biology, and a mystery of physics and chemistry, too, i.e., the puzzle of how specific reduplication of biological units occurs. Three lines of attack are being used.

(*a*) The physicist and chemist can now tell us much about the structural

and chemical nature of the bacteriophage particle itself—what it is that transforms the host cell and initiates the process of reduplication. By the same token they tell us what it is that is eventually assembled in phage synthesis. The structure of a virus particle thus contains a two-fold key to our mystery: it is a record of the events in the past which led to its synthesis; its structure likewise contains a prediction of certain events which would normally occur in the future, if and when it should encounter a suitable host cell. Just as a good machinist can tell how each part of a machine was made and assembled and how the machine should be used, so it might be thought that a good chemist or physicist should be able to tell how a virus particle was made and how it should function, if he only knew the molecular residues and their arrangement in the particles. The difficulty here is one of complexity. If a virus particle of "molecular weight" 300,000,000 be thought to be made up of 1,000,000 residues of average molecular weight 300, the minimum number of bonds between residues would be of the order of 1,000,000. Using a shorthand notation the positions of such residues could be specified in a 200 page book. As yet, however, the chemist lacks the tools necessary to specify even one of the positions of a character in such a book. Perhaps the chemist will discover that the 1,000,000 events which link the residues together fall into a few categories. Or perhaps he will learn to speak of the assembly of much larger residues than those of molecular weight 300.

(*b*) Another way to study the categories of events in virus synthesis has been to determine how the development of a virus-host complex is limited by physical or chemical interference (by x-ray irradiation, sonic vibration, by withholding essential substances, or by adding metabolic inhibitors). Or one may analyze the developing virus-host complex for molecular residues which are more or less characteristic of the virus. It may be noted that almost every fact regarding the physical or chemical nature of a virus particle becomes a tool for the experimenter who wishes to study the events concerned with virus reduplication. Certain viruses can be dissected into recognizable morphological elements, various antigenic components (88, 89) and lysins. By recording the way in which these sub-units appear during normal and altered development of the virus-host complex and the effects the sub-units have on the host, the experimenter might be able to deduce their past history and potential function.

(*c*) Finally, the more strictly biological properties of a virus are of value for the study, particularly along the lines of genetics where, from experience with higher organisms, one might assume that material sub-units or genes regulate both their self-reduplication and the elements determining the phenotypic characters of the organism. From statistical analyses of the hybrids produced in crosses between phages, one then attempts to draw inferences regarding the way in which the daughter "genes" found their places in the daughter particles. One would then like to ask, "Which of the properties of a virus are phenotypic and which are genotypic?" It is conceivable that the question is essentially meaningless. For the expression of the genotypic moiety (the passing on of characters to offspring) may well depend on

an intact phenotypic moiety (for forming a virus-producing complex with the host, say); it might be difficult, if not impossible, to make a clear distinction between the two.

Much of the productive research on virus synthesis blends all three approaches. But to the reviewer it seems that at the present time the detailed language of chemistry is too complex, while that of biology contains certain inherent ambiguities. As more phenomena are observed, a theoretical framework at some intermediate level will develop naturally and with it should come a better understanding of the relation between the bacteriophages and other forms.

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# CONSTITUENTS OF MYCOBACTERIA[1]

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In spite of the number of fractions isolated from the tubercle bacillus and other members of the genus *Mycobacterium*, relatively few of their components have been isolated in completely pure form. Practical considerations such as yields and potencies have frequently taken precedence over efforts aiming toward a goal of purity.

In 1922 the Committee on Medical Research of the National Tuberculosis Association undertook a systematic investigation of standard strains of tubercle bacilli and their culture filtrates obtained upon synthetic medium. They supported researches in many universities on problems concerned with this general plan and a summary of these researches from 1923 until 1943 was compiled by Nicolson (1). Other reviews including much of this work can be found in the book by Wells & Long (2) and the recent chapter by Long & Seibert (3). Fractions obtained by extracting the live bacilli with organic solvents were studied by Anderson and co-workers, and summaries of this work may be found in his reviews (4). Robinson (5) emphasized the significance and implications for future studies of these results. Many of the fractions isolated by Anderson were studied for their biological properties by Sabin and co-workers and these studies have been summarized by her (6). The culture filtrates were fractionated and studied by Long & Seibert and the results of these investigations have been reviewed (7).

## General

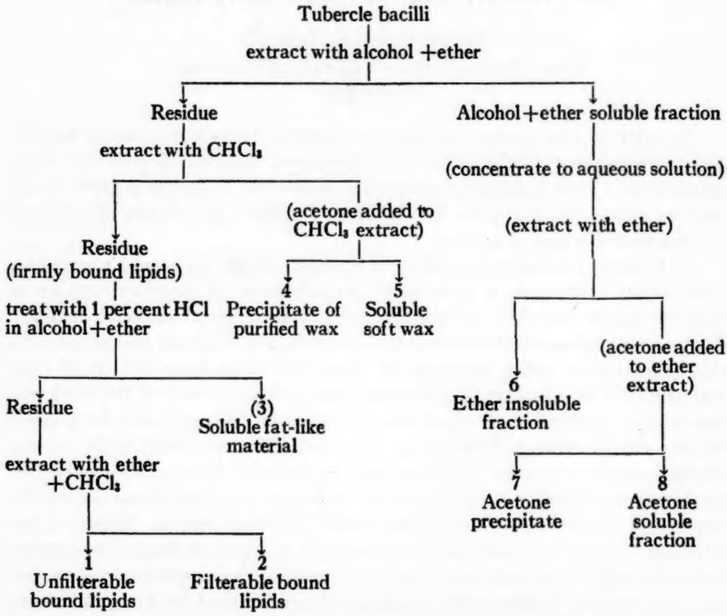
Since much of this earlier work is essential as a background to an understanding of the significance of present and future research, a brief scheme of fractionation of the bacillary cells and culture filtrate, largely taken from the works of Anderson and Seibert, is given in Tables I and II and may be helpful as a means of locating the source of any fraction under consideration. In the rest of this review emphasis will be placed upon recent and current work.

All the colloidal components in the medium are soluble, and since each has been isolated and characterized, it is possible now to survey quickly their relative quantities in any crude concentrated filtrate by means of the electrophoretic technic. Such studies have been made by Seibert (8) and show that different strains of tubercle bacilli yield varying proportions of the different components, depending upon their age of growth and many other factors. Differences due to variants of the same strain have been similarly studied by Seibert, Crumb & Seibert (9).

[1] This review covers approximately the period from March, 1941 to March, 1950.

## TABLE I

### Scheme for Fractionation of Living Tubercle Bacilli




1. Unfilterable bound lipids which on saponification yield (*a*) fatty acids; (*b*) mycolic acid; (*c*) *d*-eicosanol-2 (avian strain); and (*d*) specific polysaccharides.
2. Filterable bound lipids consisting of (*a*) hydroxy acids; (*b*) lower fatty acids; (*c*) *d*-eicosanol-2 (avian and leprosy strains); (*d*) glycerol; and (*e*) polysaccharides.
3. Soluble fat-like material of unknown composition.
4. Purified wax of 0.4 per cent phosphorus and 0.7 per cent nitrogen content. This material on saponification yields water-soluble portion-specific carbohydrates consisting of (*a*) mannose, *d*-arabinose, galactose, and traces of glucose and inositol (human); (*b*) glycerol and trehalose (timothy and avian); and (*c*) glycerol, mannose, inositol-monophosphate (bovine) and unsaponifiable portion of ether-soluble, hydroxy acids consisting of (*a*) mycolic acid (human strain); (*b*) phthiocerol (human and bovine strains); and (*c*) *d*-eicosanol-2 and *d*-octodecanol-2 (timothy, avian, and leprosy strains).
5. Soft wax which on saponification yields (*a*) glycerol; (*b*) mycolic acid; and (*c*) phthiocerol.
6. Water-soluble, ether-insoluble mixture of (*a*) polysaccharides; (*b*) riboflavin; (*c*) nonprotein nitrogen; (*d*) inorganic salts; and (*e*) proteins.
7. Acetone precipitate of ether-soluble phosphatides which on hydrolysis yield (*a*) inositol, mannose, and glucose; (*b*) glycerophosphoric acid; and (*c*) the fatty acids palmitic (saturated solid), oleic (unsaturated liquid), tuberculostearic, and phthioic (saturated optically-active liquid acids).
8. Ether and acetone soluble fraction consisting of (*a*) phthiocol, a pigment crystallizing in yellow prisms; and (*b*) fats which on saponification yield trehalose (human and leprosy bacilli), the unsaturated acids linoleic, linolenic, and oleic, the saturated liquid acids, tuberculostearic, phthioic, and a levorotatory acid (human strain), the solid saturated acids palmitic, stearic, and *n*-hexacosanoic, and some unsaponifiable material.

### Lipids

*Waxes and acid fastness.*—One of the chief constituents of the purified wax isolated by Anderson and co-workers (10) from the human tubercle bacillus was an ether-soluble hydroxy acid of very high molecular weight, designated as mycolic acid. It was acid-fast, was a saturated acid with a low dextrorotation and contained one hydroxyl and one methoxyl to one carboxyl group. Similar mycolic acids were isolated from the bovine bacillus by Cason & Anderson (11), the avian by Reeves & Anderson (12), and the phlei bacillus by Peck & Anderson (13). It was shown by Reeves & Anderson (12) and Anderson & Creighton (14) that these mycolic acids could be further

TABLE II

Important Constituents of Medium Filtrate

*Nucleic acid*
- pptd. by HCl+alcohol;
- mobility* = −12.0 or higher

*Nucleoproteins*
- pptd. at pH 4.0 (acetic acid);
- mobility = −8.6 or higher.

*Proteins*
- *A*—sol. at pH 4.0; pptd. 70 per cent alcohol; mobility = −2.6 to −3.8; colorless.
- *B*—sol. at pH 4.0; pptd. 30 per cent alcohol; mobility = −5.4 to −6.4; light color.
- *C*—pptd. at pH 4.0 (acetic acid); mobility = −6.1 to −7.3; deep amber.

*Pigments and Vitamins*

*Carbohydrates*
- *I*—sol. in 70 per cent alcohol; mobility = −1.4; mol. wt. = 6,000 to 9,000; mannose and arabinose; specific.
- *II*—pptd. 30 to 50 per cent alcohol; mobility = −1.6; mol. wt. circa 100,000; polyglucosan; antigenic; specific.

* Electrophoretic mobility in terms of $cm^2\ volt^{-1}\ sec^{-1}$.

fractionated into two principal fractions, both of which were acid-fast and very similar in their solubilities, as shown by Lesuk & Anderson (15). The mycolic acids were the only acid-fast substances that could be isolated from extracts of the tubercle bacillus and it was found by Fethke & Anderson (16) that the free carboxyl group was essential for acid-fastness, but not the hydroxyl or methoxyl groups. This fact, as well as the formula given by Anderson for mycolic acid, has been confirmed by Asselineau & Lederer (17a) and they, furthermore, isolated two isomeric, $\alpha$ and $\beta$-mycolic acids by means of chromatography (17b). Choucroun (18) was able to render defatted non-acid-fast tubercle bacilli acid-fast by adding to them in an ether suspension a solution of her acid-fast lipo-polysaccharide.

"Mycocerosic acid" is the name proposed by Ginger & Anderson (19a) for the levorotatory acid isolated from the chloroform soluble wax fractions of heated cell residues.

Phthiocerol, also obtained by them (19b) from this chloroform soluble wax, was found to be a homogeneous substance. It was studied by Ställberg & Stenhagen (20a) by means of the monolayer technic, and thought to be a very long molecule with only short side chains, and with one or more of the

polar groups near one end. These investigators also studied mycolic acid by this technic (20b).

*Phosphatides.*—In contrast to Anderson's earlier results with unheated bacilli, certain differences in the composition of the lipid fractions were found (21) in heated tubercle bacilli. A major difference was the absence of pure phthioic acid among the fatty acids obtained from the phosphatide prepared from these cells (22). However, a new substance, inositol glycerol diphosphoric acid, was isolated from this source (23). Whether the differences were due to the fact that different strains were used or to changes caused by heating was not clear.

It was also noteworthy that no phosphatide was obtained among the lipoid fractions isolated from human tubercle bacilli, H 37, grown upon a modified Long's synthetic medium in which glucose replaced glycerol (24). There was also no high melting wax, but the other lipids seemed to be similar to those found in cells grown on the standard medium. The structure of phthioic acid was given by Polgar & Robinson (25) as 3,13,19-trimethyltricosanic acid, but Cason & Prout (26) disagreed in view of its optical rotation.

*Acetone-soluble fats.*—The acetone-soluble fat isolated from heated cell residues from the preparation of tuberculin was found to contain fatty acid esters of trehalose, phthiocol, anisic acid, and the liquid saturated fatty acids, tuberculostearic and phthioic acids, similar to those found in the intact cells, and in addition several other higher liquid saturated fatty acids (27). These latter acids were found by Ginger (28), by a determination of terminal methyl groups, to contain doubly branched chains. Polgar (29) introduced a new technic for separating the fatty acids.

X-ray diffraction studied by Velick (30) supported the structure of *d*- or *l*-10-methylstearic acid for tuberculostearic acid, and Prout, Cason & Ingersoll (31) by step-wise synthesis established its structure as *l*-10-methyl octadecanoic acid. A new and improved method of synthesizing it was also reported by Schmidt & Shirley (32).

## Carbohydrates

An excellent review by Stacey & Kent (33) on the carbohydrates of mycobacteria has appeared recently and, therefore, only recent studies will be mentioned in this review.

*From the cell.*—Carbohydrates (34) as well as phosphorous-containing compounds (23) obtained from phosphatide fractions isolated from autoclaved bacterial cells differed in composition from those previously isolated from living cells and they even varied from lot to lot of the same bacilli. Free inosite, instead of manninositose, was found. Glycogen was isolated from avian tubercle bacilli by Chargaff & Moore (35) as a substance of very high molecular weight.

Haworth, Kent & Stacey obtained from moist, steam-killed cells two stable serologically specific polysaccharide fractions, a desoxyribonucleic acid derivative, and bacterial glycogen. The polysaccharide, which they

obtained mainly from the somatic part of the cell (36a), had a highly branched structure composed of mannopyranose, arabofuranose, and amino-sugar units with rhamopyranose units forming terminal residues, while the one from the outer lipoidal or waxy layer (36b) had a highly branched chain structure containing units of mannopyranose, arabofuranose, galactopyranose, and glucosamine. For more details their review may be consulted (33).

*From the medium.*—Two different serologically specific polysaccharides have been isolated from the unheated culture medium. One, designated polysaccharide I, of low molecular weight, consisting mainly of mannose and arabinose and with the biological activity of a haptene, has been described in earlier papers (39, 40) and reviews (7a, 33). The other, designated polysaccharide II, has just been described by Seibert, Stacey & Kent (37) as a large polyglucosan molecule with a molecular weight of about 100,000. It was electrophoretically homogeneous, had a rotation of +171°, and was a true antigen. It contained a small amount of lipid (8 per cent) which may or may not be an impurity, and a small amount of an amino sugar. It was a long chain molecule with very little branching, and possibly was composed of a minimum of 15 hexose units but contained no dextran, starch or glycogen. Further details concerning its structure are given by Kent (38).

## Pigments

Descriptions of the properties and isolation from the human tubercle bacilli of the pigments, phthiocol and riboflavin, as well as some carotinoid pigments from the timothy grass bacillus and the leprosy bacillus, have been given in previous reviews by Anderson (4). In 1939 he synthesized phthiocol (41). Francis *et al.* (42) isolated what they believe to be a precursor of phthiocol. Still another yellow pigment was found by Mayer (43) which increased when the avirulent acid-fast strain, No. 607, was grown in the presence of *p*-aminobenzoic acid, and the increase was shown not to be due to extra riboflavin.

Cytochrome was identified by Todd (44) in certain mycobacteria, especially *M. kalinski*, and he found two extra absorption bands, which were identified as representing coproporphyrin.

A pterin-like pigment has been identified by Crowe & Walker (45) in the tubercle bacillus by the wave length positions of selective absorption, the fluorescence characterization, chromatographic behavior, and many other reactions.

## Vitamins

Riboflavin was found by Boissevain and co-workers (46) and by Street & Reeves (47) to be identical with the yellow pigment found in synthetic culture filtrates of human, bovine, and avian strains of tubercle bacilli. Biotin (48) and also *p*-aminobenzoic acid (49) were found to be synthesized by mycobacteria, according to Landy and co-workers, and the latter also by Ekstrand & Sjögren (50). In 1946 Pope & Smith (51) confirmed these earlier reports and extended the study to other members of the B-complex.

They found in addition six other vitamins in the culture filtrates: folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxine, and thiamine, and there were significant differences between the human and bovine strains especially in their nicotinic acid contents. Vitamin assays on the whole tubercle bacillus (H 37 Rv) and the corresponding culture filtrate were made by Bird (52) who confirmed the high content of nicotinic acid. The earlier suggestions that phthiocol may be identical with vitamin K were questioned by Fernholz & Ansbacher (53).

## Enzymes

An oxidase for *l*-hydroxy acids was found by Roulet, Wydler & Zeller (54) in various strains of tubercle bacilli and other acid-fast bacteria. Later, Edson and co-workers identified two enzymes: one (55) a lactic acid oxidase in *Mycobacterium phlei*, which appeared to be an auto-oxidizable flavoprotein, and the other (56) a catalase which was found to vary widely from species to species. An enzyme in *Mycobacterium ranae* also was able to oxidize pyruvate and acetate (57). The effect of various compounds on the formation of adaptive oxidase enzymes, especially for benzoic acid, in mycobacteria, has been studied by Fitzgerald, Bernheim & Fitzgerald (58). Roulet & Zeller (59) demonstrated the presence of certain peptidases in tubercle bacilli. The culture medium of *Mycobacterium smegmatis* also contained measurable peptidase activity.

## Nucleic Acids and Nucleoproteins

Free and combined nucleic acids were found in tubercle bacilli synthetic culture medium by Seibert, Pedersen & Tiselius (60) and two methods of isolating them were proposed, one by electrophoretic separation (39), and the other by repeated precipitation by half saturation with ammonium sulfate at pH 7.0 (61, 62). Relatively pure nucleic acid was isolated by Seibert & Watson (39) from heated culture filtrates by repeated electrophoretic separation in a macro-electrophoretic cell. A maximum of 4.7 per cent protein was present in this fraction. It had no tuberculin activity.

Most of the nucleic acid, however, exists in the cell bodies rather than in the filtrate medium and can be readily released into the medium by heating the cells in the presence of water, as was shown by Seibert, Crumb & Seibert (9). This explains why considerable nucleic acid appears in Old Tuberculin (OT) preparations.

Chargaff & Saidel (63) isolated from avian tubercle bacilli a nucleoprotein, which when purified contained mostly desoxypentosenucleic acid. Pentose nucleic acid also was found in the crude nucleoprotein. Nucleic acid isolated from this nucleo-protein was found by Vischer, Zamenhof & Chargaff (64) to contain adenine, guanine, cytosine, thymine, 2-desoxyribose and ribose, but they could not find 5-methylcytosine. Boivin (65) stated that mycobacteria contain both desoxyribonucleic acid (1 to 5 per cent) and ribonucleic acid (5 to 20 per cent). No doubt the different results obtained by different authors may be at least in part due to the different strains

used. This is shown to be true in the work of Petrik (66), in which he found ribonucleic acid in only three of six strains of mycobacteria studied, but all of them contained desoxyribonucleic nucleic acid. Even *Mycobacterium phlei* contained largely the latter type of nucleic acid, in contrast to Coghill's earlier work (67).

Nucleic acid with a relatively high viscosity and, therefore, probably not much depolymerized, was isolated by Watson & Heckly (68) from the aqueous phase of bacillary bodies which had been frozen rapidly at $-70°C$. in the presence of ether. The somatic and lipoid-bound polysaccharides isolated from heat killed cells by Haworth, Kent & Stacey (36) contained respectively 2.5 and 13.7 per cent desoxyribonucleic acid.

That the desoxyribonucleic acid found by the various extraction methods may exist to a large extent in the granules or nuclear substances of tubercle bacilli was indicated by staining reactions on the cells grown in several different media in the work of Malek & Sterzl (69). Some of the literature on nucleic acid and nucleoproteins of mycobacteria has been reviewed by Stacey (70).

## Proteins

Description of the protein constituents is far more complicated than that of the other constituents, largely because their study has evolved from practical considerations involving the tuberculin reaction to the theoretical studies in protein chemistry. Thus, Old Tuberculin, which is essentially an autolytic as well as a heated aqueous extract of the tubercle bacillus, was found to contain a specifically potent protein, and isolation of the active protein, therefore, naturally began with Old Tuberculin. This approach would have been abandoned were it not for the hazard involved in working with masses of live bacilli, as well as the concern over the greater antigenicity accompanying undenatured proteins and the possibility of obtaining false sensitization with such products when used for diagnosis. The following consideration of the proteins will, therefore, carefully distinguish between those isolated from heated in contrast to unheated fractions. The chemical properties will mainly be discussed in this section and their usefulness as biological reagents will be dealt with in the sections on Tuberculin, Allergic Sensitization or Antigens.

*From heated culture filtrates.*—A practical and simple procedure for obtaining the active protein in large quantities from the heated culture filtrate was given by Seibert (71) and consisted of precipitating with trichloroacetic acid, but later it was shown that precipitation by this reagent yielded not only the protein but also the nucleic acid associated with it. However, since the two substances appeared in electrophoresis to be free of each other at a pH more alkaline than 5.0, it seemed advantageous to precipitate the protein at pH 7.0. This was done (62) and yielded a product practically free of nucleic acid. It was called PPD-S and has been used for practical diagnostic purposes (see under Tuberculin).

However, even when the nucleic acid and most of the carbohydrate were

removed from the protein, as in the case of PPD-S, the remaining protein was shown by Seibert (7c) to be a mixture of denatured and undenatured proteins which was very difficult to resolve. Therefore, most of the later researches aiming to separate these proteins have been done upon unheated filtrates, as described in the next section.

*From culture filtrates of live cells.*—Practically every method of precipitating proteins has been used for isolating the tuberculin protein and all of them yield more or less potent fractions. Among the more recent methods used have been phosphotungstic acid by Boquet & Sandor (72), absolute methyl alcohol by Daddi & Cattaneo (73), tannin by Schuberth (74), ammonium sulfate at pH 4.0 by Seibert, Pedersen & Tiselius (60) and by Bevilacqua & McCarter (75) and at pH 7.0 by Seibert (62), ammonium sulfate at pH 7.0 and then trichloroacetic acid by Schaeffer (76), and alcohol at low temperature by Seibert (8).

Protein fractions isolated in various ways were studied by means of ultracentrifugation and electrophoresis (60, 75) and molecules of different sizes and shapes were found. They ranged from 9,000 to 44,000 in molecular weight and appeared to be represented by two main groups with electrophoretic mobilities around $-3.0$ or $-6.0 \times 10^{-5}$ cm.$^2$ v.$^{-1}$ sec.$^{-1}$ in phosphate buffer, pH 7.6, $\mu = 0.1$.

Since the yields of the fractions isolated by the above procedures were relatively small, Seibert (8) investigated fractionation with alcohol at low temperature with careful control of pH, salt, and protein concentration, and obtained better resolution of the protein fractions with the different electrophoretic mobilities. Three characteristic fractions, designated as A, B and C, in the order of their mobilities, were found. The C fraction was obtained first by a simple precipitation at pH 4.0 from the concentrated culture filtrate. It had a mobility of about $-6.1$ to $-7.3$, was deep amber in color, insoluble at its isoelectric point, and always less potent as a tuberculin. The B protein fraction appeared under the same electrophoretic peak and thus had a mobility somewhat similar to that of the C fraction ($-5.4$ to $-6.4$), but it was soluble at pH 4.0 and precipitated on the addition of 30 per cent alcohol. It had a very light yellow color and was more potent than C. The A protein fraction was the slowest one, with mobility $-3.4$ to $-3.8$ and required 70 per cent alcohol at pH 4.6 to precipitate it. It was colorless in 1 per cent concentration, and of the three fractions it was the least homogeneous in electrophoresis, showing the presence of considerable carbohydrate, which was also proved chemically. How much of the carbohydrate may actually be bound to the protein has not yet been determined. Further purification is obviously required. Some further resolution of the protein fractions of low mobilities has been effected by Seibert, Crumb & Seibert (9), whereby a fraction with mobility of about 2.6 was obtained. It is highly significant that the fractions with the lowest mobilities have always been the most potent as skin-testing agents.

The chemical properties of these protein fractions, especially the most different ones, A and C, have so far been studied to some extent. For example,

the specific ultraviolet absorptions differ chiefly at Å 2,500 (8). Recent survey of their amino acid composition by Seibert & Kent (77) shows that they both contain at least ten of the ordinary amino acids. However, by chemical analyses more tryptophane and phenolic groups were found in the unhydrolyzed A than in the C fractions. Still more phenolic groups were found after hydrolysis in the A fractions but not in the C fractions, indicating that some of the phenolic groups in the A fractions were masked and were released only by hydrolysis. Since this phenomenon did not occur in the C fractions, the question is raised as to a possible relationship between these groups and the higher potency of the A fractions.

However, Fernbach & Rullier (78) did not find a strict parallelism between the potency of several of their tuberculin preparations and the extent to which these reacted with the Millon reagent. On the other hand, Kallos & Hoffman (79) suggested that tuberculin potency paralleled the content of tryptophane in their preparations.

*From live bacillary cells.*—By successive extraction of defatted bacillary cells with solutions of progressively greater alkalinity, Menzel & Heidelberger (80) obtained from the human, avian, and bovine strains a whole series of protein fractions which could be distinguished serologically.

Grönwall (81a) killed BCG organisms by freezing and drying and grinding in a ball mill at −60 to −70°C. On extraction with 0.05 to 0.10 N-NaOH he obtained a very small potent molecule with an extremely low sedimentation constant, but it was unstable on standing and aggregated. In a later study (81b) he identified in the alkaline extract three electrophoretic components, one of which he precipitated at pH 5.5 and which was probably protein. It had a very high tuberculin activity. In another study, Tiselius & Grönwall (82) found that treatment of the PPD with crystalline pepsin decreased the activity to one-tenth and the molecule was too small to study in the ultracentrifuge. Trypsin and chymotrypsin destroyed the activity completely. A potent protein fraction was also obtained by Watson & Heckly (68) from live bacillary cells ruptured by freezing rapidly at −70°C. in the presence of ether.

## Tuberculin

A review and bibliography on the *Chemistry of Tuberculin* has appeared (7d) and therefore only a brief summary of this subject will be given here. Recent suggestions that tuberculin activity may be due to an $\alpha$-$\alpha$-disubstituted fatty acid, advanced by Desbordes & Tremeau (83), or to $\beta$-hydroxybutyric aldehyde, advanced by Kasuya (84), cannot be accepted without more extensive and convincing evidence.

The need for a diagnostic purified active fraction which could be standardized and, therefore, used in exact dosages, emerged from the discrepancies, recognized by Koch himself, which continuously became evident during the use of Old Tuberculin preparations. Obvious reason for these variations in potencies and specificities is clear from recent work (9) which shows by means of electrophoretic diagrams that different raw concentrated tuberculins,

which essentially represent the composition of Old Tuberculins, vary remarkably in their proportions of polysaccharide, nucleic acid, and the different protein components, depending upon the strains of bacilli, time of growth, etc., used.

The polysaccharides and nucleic acid of tuberculin have been shown to have no tuberculin activity, while the proteins alone carry this property. Though the protein portion of tuberculin was demonstrated by electrophoresis (7c) not to be a single protein component, the need for a large batch of potent material for diagnostic purposes was so urgent that such a product containing all the protein of tuberculin was prepared in 1941 (62). It was called PPD-S, and its preparation was described under the section on proteins. This preparation could be readily duplicated in purity and potency and was shown not to cause any false sensitization in individuals being repeatedly skin-tested (85, 86).

Very large quantities of PPD have been prepared by Green (87) in England. These large quantities, used for the cattle testing program, are precipitated by trichloroacetic acid. The procedure, as modified by him, is very simple and adaptable for large scale production. PPD has also been produced by this method by Lind (88) in Denmark, but with a higher content of nucleic acid, perhaps due to the strain of bacilli used. Many of the details of the procedure were investigated by him.

In comparing different tuberculins, such as OT and PPD, some investigators have lost sight of the necessity of equating the contents of active material in the two solutions, and in consequence have misinterpreted the discrepancies which occurred. Another consideration which must not be neglected in such standardizations is in regard to the variations in sensitivities of different individuals, which are notably higher in those infected with virulent strains than with avirulent strains, such as BCG. Attention must be given also to the variations in the specificities of different protein fractions, either from different strains or from the same strain.

In the section above on PROTEINS it was noted that the A protein fraction was more potent that the C protein fraction. Moreover, variations in relative potency of the different fractions at different dilutions was evident in the studies of Seibert & DuFour (85).

The extensive studies of Palmer and co-workers have emphasized the need of a standard product. Furcolow, Hewell & Nelson (89) have shown that a dose of 0.0001 mg. PPD-S will detect 99.6 per cent of cases of known active tuberculosis. Long (90) also called attention to the work of Israel & Payne (91), showing that 95 per cent of patients with active pulmonary tuberculosis reacted even to a dose of 0.00002 mg. PPD. This low dose must be used as a first dose in areas where high sensitivity exists, in order to avoid severe reactions. However, a final dose of 0.0001 mg. could be considered adequate for clinical diagnosis.

A larger dose, such as 0.005 mg., is necessary to detect individuals with a low degree of sensitivity, such as those vaccinated with BCG or perhaps

infected with strains of low virulence. Moreover, Aronson, Parr & Saylor (92) showed that 56.7 per cent of those vaccinated with BCG needed the second dose (0.005 mg.) of PPD-S to detect their sensitivity. With the two doses 93.3 per cent were found to react. This emphasizes the high specificity of the reaction to PPD, as do also the opposite results obtained by Kahn (93) in Dutch Guiana where no reactions occurred, even to the 0.005 mg. dose, among 765 Bush negroes and Alukuyna Indians, except in 18 individuals in one village among whom was found one member who had tubercle bacilli in his sputum.

Nevertheless, the specificity of this second dose (0.005 mg.), and especially of still larger doses of PPD, has been definitely questioned by Furcolow. Hewell, Nelson & Palmer (94) because they found that everyone would react if sufficiently large doses were given. This is logical in view of the known primary toxicity of tuberculin in large doses. Thus, the necessity of a standardized product for diagnosis becomes even greater.

The fact that negative tuberculin reactions were found by Lumsden, Dearing & Brown (95) in individuals in certain areas who showed pulmonary calcification threw doubt for a time upon the specificity of the reaction, but recent studies by Palmer (96), Christie & Peterson (97), and Goddard, Edwards & Palmer (98) have shown that in most cases such individuals react to histoplasmin. The implication is, therefore, that such individuals are infected with the fungus *Histoplasma capsulatum*, or some other agent sensitizing to histoplasmin, with resultant calcified pulmonary lesions.

## Allergic Sensitization

The nature of the fraction of the tubercle bacillus responsible for the specific allergic sensitization which manifests itself as a typical tuberculin reaction is accepted to be tuberculo-protein, but by itself this protein is not able to induce so high a degree of sensitization, although it is an excellent antigen. Recent work sheds some light on this perplexing problem.

Choucroun showed that first a paraffin oil extract of heat-killed tubercle bacilli (99a), then a fraction of this extract (99b), and finally a combination of fractions including her lipo-polysaccharide complex (99c) were able to cause definite allergic sensitization to OT in normal guinea pigs.

Working with fractions of better known composition, Raffel and coworkers (100a, 100b) showed that the "purified wax" fraction, according to Anderson's scheme, and not the "soft wax" fraction, when mixed with the protein fraction, was able to induce the allergic sensitization to OT, whereas neither fraction alone was able to do so if they were highly purified.

Attempts at sensitizing animals with the tubercle bacillus or any of its fractions have usually been made by injecting the respective antigens. A recent report by Sartory & Meyer (101) indicates that the allergic sensitization can be produced also by ingestion of tubercle bacilli. Further studies on this phase may be of considerable importance in connection with the perplexing problems of cross sensitization.

## Antigens, Antibodies and Immunity

The whole live tubercle bacillus, as well as killed or defatted cells and many of the fractions of the bacillus, induce antibodies in the sera of animals into which they are injected. The question is if they are the immune antibodies desired. Most of the preparations studied as antigens have been impure mixtures of various fractions or complexes of the bacillus, as discussed below, but some studies have been made on relatively pure fractions.

*Purified proteins.*—Of the purified fractions studied, the proteins have received the most attention. These have proved to be excellent antigens with distinct specificities. For example, strains of acid-fast bacilli could be identified by means of the antibodies induced in animals by injecting the purified protein fractions isolated from these strains (102). [See a review of these studies (7a) and the work of Menzel & Heidelberger (80).] Green (87) has recently verified this and given a table of specificity units.

Even the different proteins isolated from the same strain showed differences in precipitin specificities, as seen in the work of Menzel & Heidelberger (80). Seibert & Nelson (103) and McCarter & Bevilacqua (104). Very high precipitin titers can be obtained with the sera of rabbits sensitized to the A, B and C proteins mentioned earlier (7c), and the question arises as to which, if any, of these antibodies may be the most desirable as an index of increased resistance in the animal. The caution that must be exercised in the interpretation of results can be illustrated by the following sequence of events.

It was found by Seibert & Nelson (103) that injections of certain tuberculin protein fractions into rabbits caused a marked increase in the $\gamma$-globulin fraction of their sera. This $\gamma$-globulin when isolated by means of electrophoresis, was found to give a high antibody titer to the specific antigen, and when added by Emmart & Seibert (105) to cultures of tubercle bacilli *in vitro*, inhibited their growth and also inhibited the development of tubercles on the chick membrane. Nevertheless, in spite of this apparent indication of the presence of real immune antibodies in the sera of these rabbits, they succumbed to infection as rapidly as the controls when inoculated with tubercle bacilli (117). Certainly the presence of detectable antibodies *per se* is no indication of immunity to the tuberculous infection.

The determination of the value of any of these protein fractions for serological diagnostic purposes awaits their final purification. Some promise can be offered in this respect, in view of the fact that the majority of tuberculous guinea pig sera were shown by Seibert (102) to give high precipitin titers with a relatively crude preparation. However, only 12 per cent of 51 tuberculous human sera showed precipitins with the same crude antigen. McCarter, Wisnicky & Hastings (106) also found that the sera of more than half of the cattle tested by them failed to show precipitins, even though tuberculous lesions were present.

*Purified polysaccharides.*—Many investigators have isolated carbohydrates from the bodies of the tubercle bacillus, as well as from the culture filtrates, which specifically precipitated anti-tubercle bacillus sera. Moreover, specific polysaccharides have been isolated by Anderson and co-workers

(see Table I) from the various components of the bacillary bodies, phosphatides, waxes, and bacillary residues. These are all described in detail in the review by Stacey & Kent (32), including their own polysaccharides recently isolated from the heat-killed cell residues and from lipoid-bound fractions separated by the action of basic organic substances such as urea, $\beta$-hydroxypropionamidine, etc. Among recent reports is one by Choucroun (99c, d), who isolated such a carbohydrate by hydrolysis of her lipo-carbohydrate complex.

Another different and much larger polysaccharide, called Polysaccharide II, a polyglucosan, and described in the section on carbohydrates, has recently been isolated from tuberculin (37). This proved to be homogeneous on electrophoresis and contained not more than 0.3 per cent and possibly as little as 0.02 per cent nitrogen (9). It gave a high precipitin titer with anti-tubercle bacillus horse serum and anit-BCG rabbit sera, as well as with tuberculous rabbit and human sera. Moreover, it acted as a true antigen, in that it was able to induce antibodies to itself following sensitization of normal rabbits with the purified polysaccharide alone, but it did not increase their resistance to tuberculous infection (117). Kent (38) showed that there was about 8 per cent lipid material in one of the preparations of this polysaccharide studied by him, and when this lipid was removed by extraction the purified polysaccharide still gave the same precipitin titer. The possible value of this highly purified polysaccharide as a diagnostic agent remains to be determined.

*Complexes.*—Since no relatively pure fraction has so far been found which will cause a desirable increase in resistance to tuberculous infection there have been attempts to determine whether a complex may be more effective, and thus replace the use of a living vaccine, such as BCG, the vole bacillus (107), or other attenuated bacilli, all of which have been shown to have some immunizing capacity. The hope for such a substance is supported by the fact that Opie & Freund (108) could induce some immunity with whole dead bacilli. Only complexes proposed in recent years will be mentioned in this review.

Buu-Hoï & Jouin (109) stated they were not able to confer immunity against tuberculosis with a combination of fatty acids isolated from the tubercle bacillus and protein. Nor was Raffel (100a) able to do so by injecting guinea pigs with mixtures of the various isolated and purified constituents of the tubercle bacillus, protein, polysaccharide, phosphatide, and wax, even though he was able to induce a typical tuberculin type of sensitization with a mixture of the purified wax and protein, as referred to earlier.

Choucroun (99c), on the other hand, presented evidence of increased longevity and reduced lesions in guinea pigs which had been treated with a mixture of her protein and lipo-carbohydrate complex. In view of these results it is interesting to know more concerning the chemical nature of this complex. A recent study of the constitution of her lipo-polysaccharide by Asselineau, Choucroun & Lederer (110) revealed it to be a complex mixture, in the nitrogeneous portion of which was found a tripeptide with a single

free amino group consisting of glutamic acid, alanine, and an unknown substance. This, by the chromatographic technic, appeared to correspond to an unidentified acid-stable ninhydrin-reacting substance found by Work (111) in the diphtheria bacillus. It was found also in her sensitizing fraction but not in the residues of defatted tubercle bacilli nor in tuberculin.

In a diagnostic serological test proposed by Middlebrook & Dubos (112), sheep erythrocytes were sensitized with a methyl alcohol extract of dried tubercle bacilli which had previously been extracted with 88 per cent phenol and cold acetone. Hemagglutination occurred when these sensitized cells were mixed with a limited number of sera from patients with tuberculosis or from rabbits sensitized with BCG. No chemical analyses were reported for this extract, but on serological grounds the reaction was believed to be due largely to the polysaccharide portion. The method of preparation suggests a close relationship to the methylated antigen of Negré & Boquet (113). As antigens *in vitro*, Schaeffer (114) found in the culture filtrate two polysaccharides with different specificities, a "simple" one which was able to flocculate antibacillary sera in dilutions of 1:1,000,000, and a "complex one" closely associated with the protein fraction precipitable at pH 4.0 which contained the specificity of the "simple" polysaccharide and in addition was able to fix complement. Later (114) he found that the difference in reactivity was really due to the presence of protein which was firmly bound to the polysaccharide and thus resistant to enzymatic attack, confirming earlier observations of McCarter & Watson (115).

The lipoid-bound polysaccharide complex of Haworth, Kent & Stacey (36b) which was isolated by means of urea and then precipitated with acetic acid, consisted of polysaccharide, bound lipoids, and desoxyribuncleic acid. When it was adsorbed on collodoin particles it caused a strong agglutination reaction with sera from tuberculous patients.

Bloch (116) has just reported the isolation of a lipid fraction from virulent, but not from avirulent bacilli, which is able to inhibit the migration of leucocytes *in vitro*, and which appears to be responsible for the cording phenomenon in these bacilli. It is toxic only upon repeated injection and its effect is chiefly on the capillary blood vessels. It is extracted from the bacilli by petroleum ether or paraffin oil and can be removed without loss in their viability, but a decrease in their virulence does occur.

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# MUTUALISMS IN PROTOZOA

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In this review,[1] mutualism is considered to include those relationships between two species in which both live together; the relationship is maintained during a significant part of the life cycle of both members; all, or almost all, individuals of each species live with individuals of the other species; and each lives more successfully in the presence than in the absence of the other.

The first three of these criteria are fulfilled in the relationships between flagellates and certain termites (22, 41), between ciliates and ruminants (48), and between protozoa and certain intracellular microorganisms (41, 51, 54). That a given relationship fulfills the fourth criterion of mutualism is difficult to prove, though it is often inferred if the first three are satisfied and if both members appear healthy and vigorous. Conclusive proof requires experimental evidence. The most useful experimental analysis of suspected instances of mutualism has been to separate the partners and from the characteristics of each to infer the nature of their relationship. Such experiments are the subject of this review.

## Mutualisms of Protozoa with Termites

*Zootermopsis*, *Kalotermes*, and *Reticulitermes* are the termite genera in which the role of the protozoa has been chiefly studied. *Trichonympha* is one of the prominent protozoa and *Trichomonas* is of interest because it has been grown in artificial culture. The protozoa occur in tremendous numbers, in concentrations which must be seen to be appreciated. A glimpse through a microscope of the seething mass of protozoa in the hind-gut of *Zootermopsis* affords visual confirmation of the report (40) that the protozoa constitute as much as one-third of the weight of the termite. Any hypothesis that the protozoa exert a harmful effect is incompatible with their astounding numbers and the healthy condition of the host.

*Carbohydrate metabolism.*—The fact that the protozoa ingested fragments of wood indicated that they might aid in wood digestion. Cellulose digesting bacteria have not been demonstrated in significant numbers in the termites which contain protozoa. Verona & Baldacci (64) isolated *Cytophaga* and *Cellvibrio* from *Reticulitermes* and *Kalotermes* and from their food and pellets. Ghidini (20) doubted that such forms digested significant quantities of cellulose. In later experiments, Baldacci could not demonstrate cellulolytic bacteria in defaunated termites (3).

Wood consumption by termites was correlated with the presence of the

[1] Limitations on space prevent the citation of many references.

protozoa (8), and termites deprived of protozoa by exposure to high oxygen tensions starved on a diet of wood (10). Refaunation restored the capacity to use wood. Quantitative experiments have demonstrated that the amount of wood digested by the termite alone was inadequate to meet its energy requirements and that it depended upon the protozoa (28). These results show conclusively that the termite-protozoan relationship is one of mutualism.

Buscalioni & Comes (7) postulated that glucose formed from cellulose by the protozoa was absorbed and oxidized by the termite. Trager's demonstration of a cellulase in the protozoa obtained directly from the termite and in cultures of *Trichomonas termopsidis* agreed with this interpretation (59, 60). No chemical evidence of glucose in the termite or in cultures of *Trichomonas* was obtained though gas production by a contaminating bacterium in the cultures was interpreted as evidence of glucose production in amounts too small to demonstrate with chemical methods.

A hypothesis that the protozoa not only digested the cellulose but fermented the resulting glucose was supported by the discovery that the protozoa were anaerobic and fermented cellulose to acetic acid, hydrogen, and carbon dioxide (29). Glucose was fermented to similar products (13). Acetic acid occurred in chemically demonstrable amounts in the hind-gut of termites, the wall of the hind-gut was shown to be permeable to it, and none was present in the pellets (29).

The production of hydrogen gas in faunated termites (12) indicates that this fermentation product is not completely absorbed and utilized by the host. Assuming that it is not used at all and that the cellulose fermentation by the protozoa in manometric experiments gives products in the same ratio as in the termite, the amount of acetic acid formed in the termite can be estimated from the hydrogen evolved in the manometer vessel. The amounts of acetic acid produced by individuals from several colonies of *Zootermopsis* were calculated in this way, using protozoa obtained directly from the hind-gut (34). The acetic acid was of such a magnitude that its oxidation would require approximately the amount of oxygen consumed by these termites.

This equivalence between the amount of acetic acid formed and the oxygen consumed, coupled with the demonstration of acetic acid in the gut and its absence from the pellets, together with the anaerobic habit of the protozoa, supported the hypothesis that the protozoa fermented the cellulose, forming products of which some were oxidized by the termite. The protozoa and the termite are mutually linked in the chain of biochemical events leading to the complete oxidation of the cellulose and hemicellulose in the wood. The termite comminutes the wood and transports it to the protozoa as small particles, provides a fermentation chamber, and through its own oxygen consumption, reduces the oxygen tension around the protozoa, thus aiding in the maintenance of anaerobic conditions. The insoluble carbohydrates are digested and fermented by the protozoa, providing them

with energy, and the fermentation products are absorbed and oxidized by the termite, which in this way not only satisfies its own energy requirements, but also removes the metabolic products of the protozoa which become toxic if allowed to accumulate.

A logical test of the fermentation hypothesis was to demonstrate a nutritive value of acetic acid for defaunated termites. Artificial means for continuously producing small amounts of acetic acid in the gut are not available, and the experiment has been performed by feeding salts of the acid (13, 36). These salts failed to prolong the life of defaunated termites beyond that of starved controls, indicating no utilization. However, the physiological processes suitable for absorption and utilization of the free acid might be quite inadequate for the salt, and the negative results of this experiment do not seriously weaken the fermentation hypothesis of cellulose utilization in termites.

In *Cryptocercus punctulatus*, a wood-eating roach related to termites and containing numerous protozoa, the impermeability of the hind-gut to glucose has led to the proposal (11) that glucose is absorbed from the mid-gut, the hind-gut fluid being forced between the peritrophic membrane and the wall of the mid-gut. Glucose has not been demonstrated in the hind-gut fluid of *Cryptocercus*. If, instead of glucose, fermentation products are given off by the protozoa, it may not be necessary to postulate absorption through the mid-gut. Experiments testing the permeability of the hind-gut of *Cryptocercus* to fermentation products of the protozoa would be of interest.

*Nitrogen metabolism.*—Cleveland (9) first emphasized the great nitrogen economy of termites. Hungate demonstrated that *Zootermopsis* grew on wood containing initially only 0.046 per cent nitrogen and that 50 per cent of the nitrogen consumed was assimilated (35). Quantitative analyses of growing cultures gave no evidence that nitrogen was fixed (31, 35). For each gram of nitrogen consumed, including that assimilated and that voided in the feces, 350 gm. of wood carbohydrate were decomposed. This indicates an unusually economical utilization of nitrogen.

The location of the protozoa near the mouth of the Malphigian tubules suggests that they might use the nitrogenous wastes of the termite and that a reciprocal absorption of protozoan wastes by the termite would explain the nitrogen economy (27, 44). The frugal use of nitrogen by termites does not necessarily indicate reutilization since wood decomposing fungi show even larger ratios between carbohydrates and nitrogen than do termites (30) and presumably have no mechanism for reutilization. Termites feeding on sound wood of low nitrogen content eliminate more nitrogen than they consume (31), a result not in harmony with a hypothesis of a reciprocal complete utilization of nitrogenous wastes. This idea is attractive but unsupported by experimental evidence.

The protozoa themselves rather than their nitrogenous wastes, may be a source of nitrogen for the termite. The habit of proctodeal feeding is common in termites. Grassé & Noirot (21) have described three types of procto-

deal material given off by termites: hard pellets of a characteristic shape corresponding to that of the rectal lining, softer pellets of less definite shape, and liquid material containing the protozoa. These authors state that the liquid constitutes the major portion of the proctodeal food and that the protozoa are an important source of proteins for the termite.

In order for the protozoa to be an important source of nitrogen, they must undergo cell division. Numerous mitoses in termite protozoa are found only during a brief period following refaunation after a molt of the host (1). Most of the protozoa rarely divide during the instar. In laboratory cultures, single protozoa have been observed to remain alive and active for as long as 38 days without dividing (27). Child (42) reported that protozoa were only occasionally observed in the fore-gut of *Zootermopsis*. This does not suggest that a large amount of nitrogen is obtained by consumption of the surplus protozoa by the termite. However, any small production of protozoa over and above the number needed by a particular individual could constitute a nitrogen increment becoming available to the colony through proctodeal feeding. Quantitative experiments are needed to evaluate the importance of proctodeal feeding in supplying nitrogen to the termite.

Proctodeal feeding is the means by which live protozoa are transferred from one individual to another, an important process in restoring the fauna to recently molted and to very young individuals. Transfer of protozoa in the living state would seem incompatible with their digestion and use as food. This dilemma is resolved by the observation (21) that although most of the protozoa fed artificially to termites were broken up and digested in the gizzard, some passed it intact, resisted the digestive action of the enzymes in the mid-gut, and established themselves in the hind-gut. Proctodeal feeding thus provides for transmission of the flagellates and at the same time permits their digestion. Failure of the protozoa to grow actively during an instar of the host suggests that they do not have adequate nitrogenous foods. These may be digested by the proteolytic enzymes in the mid-gut of the termite (21, 27) and be absorbed before they reach the protozoa.

Two explanations for the active growth of the flagellates immediately after the molt seem possible. (*a*) Special nitrogenous waste substances produced during the molting process may be excreted by the termite and be particularly favorable for growth and division of the protozoa whereas the usual wastes are less suitable. (*b*) The usual wastes may be sufficient to support growth and division of a few protozoa but are adequate only for maintenance when the normal population is reached. If the second explanation is correct, a mitotic flare equal to the one following a molt should occur after refaunation of artificially defaunated termites. Quantitative comparisons of mitoses under these two conditions might provide a clue to the nature of the nitrogenous materials used for growth of the protozoa.

## Mutualisms of Protozoa with Ruminants

The significance of the protozoa in the rumen has been the subject of

many investigations but has been difficult to understand. The removal of these microbes from the mammalian host has not given the same clear evidence of indispensability demonstrated for the protozoa in termites. Defaunated goats (6) and sheep (5, 69) grew as well as faunated controls and utilized cellulose with equal efficiency. This seems at first sight to negate any possible significance of the protozoa in ruminant nutrition. But the immense number of protozoa in the rumen of healthy hosts (18, 57) has made investigators loath to accept the idea that the protozoa play no role, and many interpretations of their significance have been proposed.

*Carbohydrate metabolism.*—One postulate is that although the protozoa are not indispensable for cellulose digestion, they do play a part in it, a part which may be taken over by the bacteria when the protozoa are removed. The chief reason for postulating a cellulolytic capacity in the protozoa was the fact that some of the species ingested large quantities of cellulosic material (56, 61, 68). Usuelli (63) observed as an exception that cells of barley hulls, assumed to be composed principally of cellulose, were not as readily ingested as green plant parts from hay. This exception can be explained by the fact that barley hulls actually contain very little cellulose (25). Relatively pure cellulose prepared from cotton is readily ingested by many of the rumen protozoa (32, 33).

Failure to specify the genus observed has led to conflicting views on cellulose digestion by ruminant protozoa. The larger protozoa of the genus *Diplodinium* ingest cellulosic plant parts whereas this is much less common in *Entodinium* and almost never observed in the isotrichs (33).

Cellulose ingestion by the large ruminant protozoa does not necessarily signify cellulose digestion. Cellulolytic bacteria are abundant in the rumen (37), and several investigators (18, 63, 66) have suggested that cellulose digestion within the protozoa was due to cellulolytic bacteria ingested with the cellulose, though no experimental evidence was presented. Schlottke (55) obtained protozoa directly from the rumen and washed them free of foreign material. Most of the *Entodinium* were also removed. Extracts were prepared and tested for cellulase and lichenase. A lichenase was found, but no cellulase. Failure to demonstrate cellulase was possibly due to the dilution (15×) of the extract. Weineck (67) applied chemical tests for glucose to the bodies of the protozoa and found evidence of glucose around particles of ingested cellulose. Hungate (32, 33) demonstrated a cellulase in concentrated extracts of several species of *Diplodinium* grown in clone cultures and washed free of plant parts and debris, the material ingested by the protozoa, which showed no comparable activity. The deposition of glycogen in the bodies of *Diplodinium maggii* (33) within two hr. after feeding pure cellulose was so rapid that the few bacteria ingested with the food could hardly be responsible. These evidences indicate that the cellulose digestion within the protozoa is due to enzymes elaborated within the protozoa rather than to ingested bacteria.

Starch is ingested extremely rapidly by *Entodinium* and *Diplodinium*

(18, 66). Twenty minutes after feeding 10 gm. of starch to a sheep, Usuelli (62) noted that 84 per cent of the grains were contained within the bodies of the protozoa, and at 6 hr., the percentage was 94. When 50 gm. were fed, 25 per cent of the grains were within the protozoa at 20 min. and 40 per cent at 6 hr. Ferber (18) noted that starch grains not ingested by the protozoa were digested, but more slowly than those within the protozoa, and concluded that an amylase was formed by the protozoa. However, according to van der Wath & Myburgh (66), uningested starch grains disappeared as rapidly in a defaunated as in a faunated animal. Schlottke (55) found more amylase in the extracts of rumen protozoa than in rumen bacteria. The equal rate of disappearance of starch in the faunated and defaunated animals of van der Wath & Myburgh does not necessarily contradict the view that the amylolytic activity within the protozoa is stronger than that outside. If in the faunated rumen, relatively more starch is digested within the protozoa than outside, the amylase within must be more concentrated in order to digest the starch within the same period. McAnally & Phillipson (47) point out that the rapid deposition of glycogen after starch ingestion is more consistent with formation of an amylase by the protozoa than with starch digestion by ingested bacteria. As in the case of cellulose digestion, the reported observations and experiments indicate that starch digestion in the protozoa is due to enzymes formed within them.

A hypothesis that the glycogen deposited in the bodies of the rumen microbes is their chief contribution to the carbohydrate nutrition of the host has been proposed (2, 66). The host receives the carbohydrate when the microbes and their contained glycogen are digested in the abomasum. All of the carbohydrate digested within the protooza cannot be utilized in this way because part of it must be fermented to supply energy to the protozoa (16, 36). The proportion of food available to the host as glycogen depends on the rate of carbohydrate storage as compared with its fermentation. This rate is probably not the same for all protozoa which digest cellulose. In *Diplodinium maggii*, the amount of glycogen present is usually much greater than in *D. neglectum*, using the intensity of the iodine reaction as an index of quantity. It seems probable that the amount of carbohydrate stored as glycogen is less than the amount fermented by the protozoa. The large quantities of stored glycogen, rather than representing the major part of the food used, may reflect the active fermentation and be an adaptation which permits the protozoan to continue fermentation when carbohydrate food is not readily available (56). The importance in ruminant nutrition of microbial storage substances as compared with fermentation products is susceptible to quantitative experimental analysis, and such a study is needed for a reliable evaluation of the relative importance of microbial polysaccharides and microbial fermentation products in ruminant nutrition.

*Nitrogen metabolism.*—In addition to the role of some of the protozoa in carbohydrate utilization, it has been postulated (23, 48, 57) that the proteins of the protozoa are more easily digested and more nutritious than the

proteins in the plant material consumed by ruminants and that assimilation of plant and bacterial proteins into protozoa is an aid to the host. Johnson *et al.* (38) found that the proteins in the rumen bacteria and protozoa had the same biological value. This is also suggested by the failure of defaunation to diminish the growth of the host.

The importance of the protozoa in the use by ruminants of simple nitrogen compounds such as asparagine, urea, and ammonia has been tested by Johnson *et al.* (38), who found a biological value of 49 for urea fed to defaunated sheep. This is lower than the value of 62 reported by Harris & Mitchell (26) for faunated sheep, but results of urea feeding depend so much on the other foods present that this comparison between two experiments by different investigators is not valid. The well known ability of many bacteria to assimilate the nitrogen in urea or ammonia makes it highly probable that they, rather than the protozoa, are responsible for the utilization of these substances in the rumen.

From the preceding discussion of the rumen protozoa, it should be evident that the activities which they perform can also be accomplished by rumen bacteria and according to the results of defaunation experiments, can be accomplished equally well. Under these circumstances, should the protozoa be considered as mutualistic symbionts of the ruminant? In discussing this question, it should be emphasized that the ruminant has evolved a highly specialized mechanism for utilizing crude fibre as food. Anaerobic microbes in the capacious paunch convert cellulose and hemicellulose to products assimilable by the host (16). The ruminant has become so adapted to this fibrous food that continued health requires the feeding of at least a certain amount of roughage (66). The environment set up by the host is unique, and most of the protozoa and possibly some of the bacteria are found nowhere else (37, 52). The host thus benefits the rumen inhabitants by providing a favorable environment.

In the ruminant, no mechanism has evolved for separating the nonfibrous foods which it can digest from the fibrous materials which must be fermented. The readily used foods undergo the same fermentation as fibrous materials and have a similar food value. Krogh *et al.* (43) estimated that 4.6 per cent of the available energy was liberated in the microbial fermentation. The fibrous foods suffer a similar loss due to fermentation, but the energy available to the host through oxidation of the fermentation products of the fiber more than offsets any loss due to fermentation of both fibrous and nonfibrous materials. The adaptation of the ruminant to a preliminary microbial fermentation of its food is thus advantageous.

Not only does the ruminant absorb and utilize the products of microbial carbohydrate fermentation but it also uses the microbes. Gruby & Delafond (23) showed that the protozoa disappeared during passage of food through the alimentary tract; the authors concluded that the protozoa were digested and absorbed by the host. The ruminant may be likened to a plankton feeder with the added adaptation that it rears the planktonic forms and

utilizes not only their cells but also the fermentation products of their growth.

The ruminant cannot select and maintain a mixed culture of a few microorganisms in the rumen. Many are able to survive, and the rumen is a veritable microcosm with innumerable relationships between the diverse inhabitants. Any microbe in the rumen may participate in the planktonic food chain leading to the host. The significance of each depends upon the kind and extent of its participation.

Considering the problem in this light, the protozoa are significant because they compose an appreciable fraction of the total microbial protoplasm. Ferber (18) centrifuged the protozoa to separate them and found that in the sheep they constituted 1/20 of the weight of the rumen contents and in the goat 1/12 of the weight. The nitrogen in the protozoa was 10 to 15 per cent of the total nitrogen in the sheep rumen and 20 per cent of that in the goat. Schwarz (57) separated the protozoa by filtration and from analyses concluded that they contained about 20 per cent of the nitrogen in the rumen. Mangold & Schmitt-Krahmer (49) estimated the relative amount of nitrogen in the protozoa by comparing the nitrogen content in various fractions of faunated and defaunated rumina and concluded that there was practically no nitrogen in the protozoa. Their method seems less reliable than the direct measurements. Ferber & Winogradowa-Fedorowa (19) calculated the amount of nitrogen supplied the host by the protozoa as about 2 per cent of the total, but this figure was shown by Mowry & Becker (50) to be based on the erroneous conclusion that if 7 per cent of the protozoa were in the process of mitosis, only this percentage divided each day. A division rate of once per day in flask cultures (32, 33) suggested at least this rate of division in the rumen. Johnson *et al.* (38) stated that the number of protozoa in the rumen doubled in the first 16 hr. after feeding. Assuming as a minimum rate that they divide at least once per day and that there is a continuous draw-off of rumen contents into the omasum, it can be calculated (32) that the protozoa digested by the host per day would be equal to 69 per cent of those in the rumen. This yield of protozoan nitrogen to the host is 10 times the value of 7 per cent used by Ferber & Winogradowa-Fedorowa, and if substituted in their calculations, gives 20 per cent as the fraction of the total nitrogen supplied by the protozoa. It may be concluded that about 20 per cent of the nitrogen used by the ruminant is obtained in the form of protozoa.

Ferber (18) first noted the correlation between the number of rumen protozoa and an increased assimilation of nitrogenous foods (50, 66). Animals which required more nitrogen due to pregnancy or lactation contained twice as many protozoa per milliliter as other adults and young growing animals showed equally high counts. Although Ferber interpreted this as evidence that the protozoa were essential in protein utilization, it is more probable, as Mowry & Becker (50) deduced, that the increased protozoa result from the increased food ingestion determined by greater appetite. Hale *et al.* (24) reported that in adult ruminants without needs for extra nitrogen, the

microbial attack on food was complete within 12 hr. after ingestion, and Johnson *et al.* (38) found that methane production was greatest during the first nine hours after feeding. This suggests that in these animals, the actual microbial growth was less than the potential growth, since it was limited by the rate of food ingestion. In animals with greater appetite, the more rapid food passage, if it maintained better conditions for growth of the entire microbial population, should lead to the development of greater numbers. This microbial adjustment for meeting the increased food demands of the host would be automatic.

The successful utilization of fibrous foods by defaunated ruminants shows that the rumen bacteria alone can successfully accomplish the microbial role in the mutualistic relationship. But the equally satisfactory utilization when protozoa are an important microbial component shows that their presence does not diminish the successful utilization of ruminant food. Since they are so numerous, it must be concluded that in addition to the action of some of them in digesting starch and cellulose they play a significant role in the food chain leading to the host.

## Mutualisms of Intracellular Microbes with Protozoa

*Mutualisms with algae.*—Experiments on these relationships have been performed almost exclusively with *Paramecium bursaria*. Pringsheim (54) eliminated the zoochlorellae by keeping the protozoa in the dark with yeast as food and then holding them in a saturated solution of calcium sulfate at 30°C. When fed on yeast in the dark, the colorless protozoa grew and divided at about the same rate as those containing zoochlorellae. A few algal cells remained alive in some experiments and must have received all their nutriment from the protozoa.

When placed in a purely inorganic solution in the light, only the protozoa containing algae lived for an extended period (54). Though no organic nutrients were added to the medium used in the light culture, some bacteria were present. Loefer's (45) finding that bacteria-free *P. bursaria* with algae would grow in peptone but not in an inorganic medium suggests that in Pringsheim's experiments the bacterial contaminants may have supplied some essential factors.

Loefer (46) was able to grow the zoochlorellae of *P. bursaria* on beef extract agar containing either starch or peptone and nitrate. Addition of 0.5 per cent glucose or 0.2 per cent sodium acetate improved the growth. It may be surmised that when the algae are maintained by the protozoan (as in the dark with yeast as food), they derive from it complex nutrients equivalent to those obtained from this pure culture medium. The pure culture of the zoochlorellae resembled *Chlorella ellipsoidea*, but confirmation of this identity by infecting protozoa with stock strains of *C. ellipsoidea* was not attempted.

The media used for pure cultures of *P. caudatum* (39) and for *P. aurelia* (65) included such complex substances as yeast autolysate, lettuce infusion,

and heat killed *Aerobacter* cells. If the food requirements of *P. bursaria* are similar, its ability with its algae to grow in much less complex media suggests that in the light and in the absence of particulate food, the protozoan obtains complex nitrogenous nutrients from the algae. Under almost completely autotrophic conditions of nutrition, a considerable quantity of materials must pass from the alga to the protozoan. It would be interesting to determine whether similar amounts are released from an autonomous algal cell.

Doyle & Doyle (14) noted that in the dark, starch granules disappeared and crystals of calcium oxalate accumulated in the zooxanthellae of *Orbitolites* and to some extent in the surrounding cytoplasm of the protozoan. In the light, the crystals disappeared and starch formed. The observations suggest that calcium oxalate might be a respiratory product whose insolubility permits retention until light is again available to reduce it. That reduction caused disappearance of the oxalate was indicated by disappearance of the crystals in light or dark when the cells were kept in a hydrogen atmosphere. Storage of calcium oxalate would be advantageous if carbon dioxide were not available. An increased starch formation in light when the carbon dioxide tension in the sea water was increased suggests that the latter was a limiting factor. If calcium were more readily obtained than carbon dioxide, the storage of calcium oxalate would permit more subsequent photosynthesis than if carbon dioxide were required. Proof that the calcium oxalate has such a significance requires more experimentation, which may also disclose whether mutualism is concerned. It is conceivable that, under certain conditions, carbon dioxide might be stored in the form of calcium carbonate.

*Mutualisms with nonphotosynthetic microbes.*—Almost all investigators who have studied termite protozoa have reported bacteria in the endoplasm (7, 41). Pierantoni (51) suggested that symbiotic bacteria within the termite protozoa fixed atmospheric nitrogen. This was not supported by experimental data, and the absence of nitrogen fixation in colonies of *Zootermopsis* (31, 35) shows that at least in some termites it does not occur.

Although cellulolytic bacteria in the gut of termite species containing protozoa have not been demonstrated to play a role in cellulose digestion, the common occurrence of cellulase among bacteria has led to proposals that bacteria within the cellulose digesting protozoa were responsible (3, 7, 20, 51, 61, 66). Mangold (48) describes such a situation as *Symbiose zwischen den Symbionten der Wiederkäuer*. If they provide favorable conditions for endocellular cellulolytic bacteria and thus aid the ruminant, it would seem that the protozoa should be classed as helpful.

There is little evidence for or against the hypothesis that cellulose digestion within the protozoa is due to bacteria. The morphological basis for originally suspecting cellulose digestion in the protozoa of termites and ruminants was the fact that they ingested large quantities of cellulosic food. A morphological basis for suspecting cellulose digestion by intracellular bacteria would be a location near the cellulosic food particles. Descriptions of the endocellular bacteria within the protozoa of termites and cattle do not report an intimate contact with the wood.

A bacterial cellulase could be elaborated and given off by bacteria located in the protozoan at points distant from the food. Such bacteria might be expected to live in a more intimate relationship with the protozoa than if they directly attacked the fibers, and it might be expected that they would have undergone nutritional modifications that might make them difficult to culture. As has been pointed out (15, 58), such bacteria would be hardly distinguishable from mitochondria or other cytoplasmic bodies. Failure to obtain cultures of cellulolytic bacteria from termite protozoa does not preclude their presence. However, successful culture of intracellular cellulolytic bacteria and proofs that they occur in considerable numbers in the protozoa would be an extremely convincing evidence of their existence. *Trichomonas termopsidis* does not appear to contain any typical bacterial cells, yet the experiments of Trager (59) show that it contains cellulase. Also, *Entodinium* contains bacteria-like bodies, yet there is no evidence that it contains cellulase.

A mutualism between *Paramecium aurelia* and a possibly microbic habitant of its cytoplasm has been disclosed in a series of admirable experiments by Sonneborn (58). The "killer" factor discovered and described by Sonneborn and at first regarded as a cytoplasmic factor, now appears from the work of Preer (53) to possess many characteristics of a microorganism such as a rickettsia. Lacking explicit knowledge of its nature, this factor has been called kappa. The paramecium harbors the kappa bodies, and if a sufficient number are present, the protozoan is protected from a toxic substance, paramecin, given off from paramecia containing kappa. Protozoa not containing kappa or with only a few are killed by paramecin. The presence of kappa thus confers a survival advantage on the protozoan. If kappa is an organism, their relation is one of mutualism.

These experimental results emphasize the possibility that symbiosis has played a significant role in the evolution of existing life. Symbiotic phenomena are so widespread in nature at the present time that it is reasonable to suppose they were similarly abundant in past eras. The importance of competition in nature has been stressed in the law of natural selection almost to the exclusion of mutualism. Yet mutualism explains some phenomena of evolution. One of the prominent evolutionary steps leading to increasing complexity in living organisms has been a remaining together of like parts with subsequent differentiation and integration into a higher unit. Multicellular organisms thus evolved from single cells. The relationship between the single cells of a multicellular organism is similar to mutualism except that the cells are assumed to have been originally of one species. Segmentation and metamerism are a modern record of an ancient integration of multicellular units to form still more complex organisms. Individual insects or humans joined into societies represent still a higher category (17). This process, when applied to units below the cell, suggests that the cell arose by integration of simpler units, and the similarities between genes and viruses suggest that chromosomes may have originated as linear colonies of viruses.

For the present review, the significant point is that increasing complexity

may arise not only by integration of identical units of the same level of organization, but also through integration of unlike units of the same or different levels, particularly if mutually advantageous. Speculations in this field, in order to be fruitful, should lead to experiment. Sonneborn has initiated experiments which have already proved illuminating. As Beadle (4) points out, further studies on the nature of self duplicating cytoplasmic units may be extremely helpful in understanding fundamental biological phenomena.

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# BACTERIAL METABOLISM[1]

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The year of 1949 was marked by extensive investigations in all phases of bacterial metabolism. Owing to the profuse and heterogeneous nature of the resulting literature, it was not feasible to attempt a complete review. In this review, therefore, an effort will be made to evaluate critically certain reports and to leave to the abstracting journals the compilation of summations. The omission of a particular study in no way is meant to reflect on the quality of that work.

## Intermediary Carbohydrate Metabolism

*Phosphorylation*.—Meyerhof (1) has ably demonstrated that the Harden-Young effect[3] obtained in the fermentation of glucose with yeast extracts and dried cells is due to the inactivation of adenosinetriphosphatase. Preparations of quickly dried brewer's yeast which still possessed adsorbed adenosinetriphosphatase were obtained. Fermentation of glucose by these preparations did not exhibit the Harden-Young effect. The addition of adenosinetriphosphatase inhibitors, i.e., octyl alcohol, phenylurethane, and toluene, to such a fermentation produced the typical Harden-Young effect. A preparation obtained by disruption of dried yeast with sonic vibration showed a partial Harden-Young effect which was due to partial inactivation of the adenosinetriphosphatase. Addition of the inhibitors to this preparation produced a full effect. These facts show conclusively that destruction of adenosinetriphosphatase by manipulation of the yeast cell or by addition of specific inhibitors brings about the Harden-Young effect. Previous arguments against the occurrence of phosphorylative decomposition of glucose in the fermentation by living yeast are no longer tenable in view of these results.

Leloir *et al.* (2) isolated *Escherichia coli* from solutions of glucose-1-phosphate in which appreciable amounts of glucose diphosphate appeared upon storage. It was found that intact cells of *E. coli* can convert glucose-1-

[1] This review covers approximately the period from January, 1949 to January, 1950.

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[3] The Harden-Young effect is characterized by two phenomena: (*a*) stoichiometric accumulation of hexose phosphates simultaneously with the fermentation of sugars and (*b*) a sudden decrease in the rate of fermentation after the free phosphate or the free sugar is exhausted. The effect is not obtained with living yeast but only with preparations of yeast obtained by various procedures.

phosphate to glucose diphosphate in yields as high as 4 per cent of the initial amount of monophosphate added. The proposed mechanism was:

$$2 \text{ glucose-1-phosphate} \rightarrow \text{glucose-1,6-diphosphate} + \text{glucose} \qquad \text{I}$$

which involved a transfer of phosphate from position-1 of glucose-1-phosphate to position-6 of another molecule of the same substance. A transphosphorylation involving adenosinetriphosphate (ATP) and glucose-1-phosphate did not appear to be involved. Cell-free extracts were prepared which actively catalyzed the reaction. The molar ratio of glucosediphosphate /glucose obtained with these extracts was much less than one, indicating the presence of other enzymes. Leloir *et al.* also observed that inorganic phosphate was liberated in amounts approximately equivalent to the reducing substances formed. Indications were obtained that the increase in glucose and inorganic phosphate was due to phosphatase activity. With intact cells of *E. coli*, glucose-1-phosphate and glucose were fermented at equal rates, whereas glucose diphosphate was decomposed very slowly. It remains to be determined by what mechanism glucose diphosphate is metabolized. If permeability phenomena are not involved in these experiments, it is difficult to assign any role to glucose diphosphate other than that of an intermediate in the phosphoglucomutase reaction (3).

Wilkinson (4) obtained evidence that the first step in the adaptive fermentation of galactose by top yeast was its conversion to galactose-1-phosphate under the influence of a galactokinase. Galactose-6-phosphate was not an intermediate since cell-free extracts could not ferment it. Fructose-1,6-diphosphate was formed from galactose and it was believed that glucose-6-phosphate was an intermediate. The mechanism of this transformation was elucidated by Caputto *et al.* (5) who investigated reaction II as catalyzed by a purified enzyme preparation obtained from *Saccharomyces fragilis.*

$$\text{galactose-1-phosphate} \xrightarrow[(a)]{} \text{glucose-1-phosphate} \xrightarrow[(b)]{} \text{glucose-6-phosphate} \qquad \text{II}$$

Reaction (*b*) was brought about by phosphoglucomutase whereas reaction (*a*) was found to require a thermostable factor present in mammalian liver and commercial yeast. The same investigators (6) have recently identified the factor as uridinediphosphate glucose.

Barker & Lipmann (7) investigated the role of phosphate in the decomposition of sugars and polyalcohols by dried cells of *Propionibacterium pentosaceum.* Transphosphorylation reactions from ATP or phosphopyruvate to glucose, arabinose, glycerol, and erythritol were readily catalyzed by these preparations. No transfer was observed with sorbitol or mannitol. Inorganic phosphate was oxidatively incorporated into phosphoglyceric acid during the oxidation of glycerol with pyruvate or fumarate as the hydrogen acceptor. Iodoacetate inhibited the oxidation and the uptake of inorganic phosphate. Erythritol was oxidized in the presence of pyruvate with the uptake of inorganic phosphate and formation of a difficultly hydrolyzable ester which apparently was not phosphoglyceric acid. This ester was postulated to be a

four carbon acid phosphorylated on the 4-position by analogy with the 3-phosphoglyceric acid derived from the oxidation of glycerol. Sorbitol was oxidized in the presence of pyruvate with the uptake of inorganic phosphate and formation of stable phosphate esters. Phosphoglyceric acid was one of the components. Since sorbitol is not phosphorylated by transphosphorylation reactions, the view was taken that sorbitol is first oxidized directly to a sugar which is phosphorylated and then oxidized with the generation of an energy-rich phosphate bond. Glucose was oxidized in the presence of pyruvate or fumarate with the uptake of between 1.5 and 2 moles of inorganic phosphate per mole of glucose decomposed. Phosphoglyceric acid was the only ester obtained. With arabinose, the data indicated that only one mole of phosphate was esterified during its oxidation in the presence of fumarate, the ester obtained being phosphoglyceric acid. Such results are compatible with the view that the pentose-monophosphate is cleaved to triose phosphate and an unphosphorylated $C_2$ fragment. Inositol was not phosphorylated directly by ATP and was oxidized without the uptake of inorganic phosphate. Approximately equivalent amounts of acid and reducing sugar were formed during the oxidation. Pyruvic acid was fermented in the presence of sodium fluoride with the esterification of phosphate. Most of the inorganic phosphate utilized was accounted for by an increase in easily hydrolyzable phosphate. This ester could not be identified as phosphopyruvate or acetyl phosphate. The importance of phosphate in the metabolism of various sugars and polyalcohols has been reaffirmed. With most of the substrates studied, transphosphorylation with ATP appeared to be necessary. Subsequent oxidations involved the uptake of inorganic phosphate to create energyrich phosphate bonds.

*Fermentation and oxidation.*—Stokes (8) investigated the report (9) that the fermentation of glucose by *E. coli* is a typical lactic acid fermentation (homofermentative). Such results are not in accordance with the work of several investigators who have invariably found more than two equivalents of acid produced per mole of glucose utilized. In the present work, two types of cell suspensions were used: (*a*) cells grown aerobically which did not possess hydrogenlyase activity and (*b*) cells grown under reduced oxygen tension which had hydrogenlyase activity. The fermentation of glucose by resting cells of the former type in sodium bicarbonate buffer, pH 7.1, gave approximately 0.8 mole of ethanol and of acetic acid, 1.2 moles of formic acid, 0.2 mole of lactic acid, and 0.4 mole of succinic acid per mole of glucose fermented. Within experimental error, these results give a proper carbon balance and redox index. The quantity of acid measured manometrically in the presence of sodium bicarbonate agreed with that determined chemically. Carbon dioxide recoveries in these manometric experments indicated no net production nor assimilation of carbon dioxide. Owing to these carbon dioxide balances, the author stated that these results " . . . show conclusively that $CO_2$ was not assimilated during the fermentation." This was taken as evidence that succinate was not produced by condensation of carbon dioxide with pyruvate to yield oxaloacetate with a subsequent reduction to succinate.

The hypothesis that formate condenses with some three-carbon intermediate for the production of succinate was presented. As evidence in support of this hypothesis, it was pointed out that the molar sum of the formate which presumably had condensed to form succinate and the formate found as a product was equal to the molar sums of the two-carbon products, ethanol and acetate. Apparently the two-carbon compounds and the formate were produced by the phosphoroclastic reaction. It would appear unwise to disregard carbon dioxide fixation as a mechanism for succinate formation on the basis that there was no net assimilation of carbon dioxide. The possibility remains that the net production equalled the net assimilation under such conditions. The methods employed would not detect such a balance. When the fermentation was conducted at a lower pH in bicarbonate buffer, the yield of lactic acid was the same. However, fermentation of glucose by *E. coli* in phosphate buffer resulted in increased lactic acid formation at low pH values as compared to high values. The decrease in lactic acid yield at high pH values was accompanied by an increase in ethanol and volatile acids, the latter consisting chiefly of formic acid. If one calculates carbon recoveries and redox indices from the data presented, unsatisfactory values are obtained. This suggests that carbon dioxide and/or hydrogen were produced which the data did not reveal.

Cohen-Bazire & Cohen (10) obtained evidence for the direct transformation of fumarate to oxaloacetate without the intermediate formation of malate by cell suspensions of *Clostridium saccharobutyricum*. In the fermentation of fumarate and oxaloacetate, more generous yields of volatile acids (acetate and butyrate) were obtained than in the fermentation of malate. In addition, the molar ratio of acetate to butyrate varied from 2:1 to 1:1 when fumarate and oxaloacetate were fermented, whereas, in the fermentation of malate the acetate to butyrate ratio was much greater than two. The fermentation of fumarate was not influenced by the presence of congo red, which is supposedly an inhibitor of fumarase. Owing to the nonspecific action of this dye, particularly with cell suspensions, this result cannot be considered conclusive. The formation of malate by the action of fumarase on fumarate in the presence or absence of the dye was not tested. A further difference in the behavior of malate, compared with fumarate and oxaloacetate, was observed when these substances were fermented in the presence of equimolar quantities of hydroxylamine. Fumarate and oxaloacetate, but not malate, served as hydrogen donors for the reduction of hydroxylamine to ammonia. Hydroxylamine inhibited the production of volatile acids from the three dicarboxylic acids. Aspartate was detected by paper chromotography as a product of the fermentation of fumarate and oxaloacetate in the presence of hydroxylamine, but was not detected with malate. Two mechanisms were proposed for the production of ammonia: (*a*) the direct reduction of hydroxylamine by the oxidation of fumarate and (*b*) the reduction of the oxime of oxaloacetate to aspartate during the oxidation of fumarate followed by an oxidative deamination of aspartate to oxaloacetate and ammonia. The proposed mechanism of reduction of hydroxylamine via the oxime of oxalo-

acetate was discussed in relation to nitrogen fixation. [See (11) for role of hydroxylamine in nitrogen fixation.]

Lerner & Mueller (12) demonstrated that the poor glucose fermenting ability (alcoholic fermentation) of a mutant strain of *Clostridium tetani* cultivated on an iron deficient medium was due to the lack of glutamine in these cells. Added glutamine restored the fermentative ability. Cells grown in an iron-rich medium fermented glucose at an optimal rate. The presence of iron was supposedly required for the elaboration of glutamine by the growing cells. It would be interesting to examine this phenomenon further in view of the results of Muntz (13), who showed that ammonium or potassium ions are required for maximum fermentation by yeast zymase in addition to heretofore recognized cofactors.

Cochrane & Dimmick (14) identified succinic and lactic acids as metabolic products from an actively growing culture of *Streptomyces coelicolor* with glucose and small amounts of asparagine in the medium. Considerably more succinic acid than lactic acid was present and a small amount of an unindentified keto acid was produced. No volatile acids were detected. *Streptomyces griseus* and *Streptomyces reticuli* formed very little acid from glucose.

Miller *et al.* (15) investigated the effect of homologous rabbit immune serum on the activity of a purified preparation of yeast hexokinase. Complete inhibition was obtained if the immune serum was added to the hexokinase prior to the addition of glucose and ATP or after phosphorylation had begun. The yeast hexokinase antiserum had no inhibitory effect upon rat brain hexokinase. Rabbit antiserum prepared by intramuscular injection of yeast hexokinase in mineral oil gave high precipitating and complement fixing titers but did not inhibit the yeast hexokinase reaction. It is conceivable that the production of specific yeast hexokinase antibodies occurs only if active centers of the enzyme are intact. The specificity was further demonstrated by showing that rabbit antihyaluronidase serum did not inhibit the yeast hexokinase reaction.

Kun & Abood (16) studied the properties of the succinoxidase present in endotoxin preparations from *Salmonella aertrycke*. The enzyme did not contain cytochrome oxidase, but catalyzed the reduction of cytochrome-*c*. It was inhibited by iodoacetamide and malonate. In the presence of reduced methyl violet, the preparation reduced fumarate to succinate. This reduction was not inhibited by malonate or iodoacetamide. The enzymes involved appeared to be flavoproteins which, when reduced, reacted with oxygen to form hydrogen peroxide. The preparation also had catalase and peroxidase activity. In another publication, the same investigators (17) studied the mechanism of inhibition of glycogen synthesis in rats by endotoxin preparations from *S. aertrycke* and Type I meningococcus. The amount of liver glycogen in rats fed glucose was significantly higher than in animals fed glucose and injected with either of the endotoxins. The same endotoxin preparations markedly inhibited pyruvic acid oxidase from rat and rabbit liver. It was suggested that the inhibition of pyruvate oxidation plays an important role

in the toxic effects of endotoxins. No correlation was made between this inhibition and the succinate oxidase activity of the preparation mentioned in the previous paper.

Pappenheimer & Hendee (18) compared the succinoxidase system of beef heart muscle and *Corynebacterium diphtheriae*. The former contained relatively large amounts of cytochrome-*c*, cytochrome oxidase, and the factor which mediates reduction of cytochrome-*c* by cytochrome-*b*. The bacterial system contained far more succinicdehydrogenase activity and cytochrome-*b*, but only traces of cytochrome-*c* and cytochrome oxidase. The rate of oxidation of succinate in the bacterial preparation depended upon the rate of autoxidation of cytochrome-*b*. Attempts to resolve the succinicdehydrogenase and cytochrome-*b* failed. The view that the two components were identical in both beef heart and bacterial preparations was expressed.

*Pentose metabolism.*—Cohen (19) investigated the hypothesis that uronic acids are utilized by a preliminary decarboxylation to their homologous pentoses, i.e., D-glucuronic acid to D-xylose and D-galacturonic acid to L-arabinose. The technique of simultaneous adaptation was employed using bacteria continually maintained in their exponential phase in a medium of mixed substrates under conditions minimizing selection while permitting adaptive enzyme formation. If the individual steps of uronic acid utilization are under adaptive enzymatic control, then growth on a medium that contains uronic acid will produce cells that should be simultaneously adapted to the homologous pentose if the latter is an intermediate step in the utilization of uronic acid.

A strain of *E. coli* capable of growth on D-galacturonic acid and D-glucuronic acid was used. When this organism was grown on glucose and subcultured into a fresh medium containing glucose, exponential growth began immediately. With galacturonic acid, glucuronic acid, L-arabinose, D-xylose, and D-ribose, there was a lag period of about 40 min. and the mass doubling times for these substrates was considerably longer than for glucose. In mixtures of glucose and uronic acid, exponential growth occurred until the glucose was exhausted, and then exponential growth continued at a new rate characteristic of the uronic acid. The formation of adaptive enzymes for uronic acid utilization apparently took place during growth on glucose. In mixtures of glucose and pentose, bacterial growth was a two-step process, i.e., the diauxie phenomenon (20) was observed. In the first phase, the organisms grew on glucose. After the latter was exhausted, a lag period followed during which adaptive enzyme formation for pentose took place. Apparently glucose inhibited the formation of adaptive enzymes for pentose utilization. Proper ternary mixtures of uronic acids, glucose, and pentose gave organisms maximally adapted to pentoses. In such mixtures, the glucose-pentose diauxie was eliminated. Cells adapted in this manner to uronic acids and pentoses were tested for their ability to oxidize and ferment the homologous pentose or uronic acid. For example, cells adapted to glucuronic acid immediately took up extra oxygen when glucuronic acid was added, but the rate of oxygen uptake in the presence of xylose was the same as in the absence of substrate.

Similarly cells which were adapted to xylose oxidized xylose immediately but did not oxidize glucuronic acid. Similar results were obtained if acid production was measured under anaerobic conditions instead of oxygen consumption. Adaptation to a single pentose produced cells completely specific for that pentose with respect to both oxidation and fermentation. However, cells adapted to either glucuronic or galacturonic acids oxidized or fermented both uronic acids. It was concluded that the uronic acids are not metabolized by way of their homologous pentoses. The following hypothesis was presented:

$$\begin{tikzcd} \text{Uronate} \arrow[r, "(a)"] & \text{uronate phosphate} \arrow[d, "-CO_2"] \\ \text{Pentose} \arrow[r, "(b)"] & \text{pentose phosphate} \arrow[d, shift right, "O_2"'] \arrow[d, shift left, "N_2"] \\ & \text{undetermined products} \end{tikzcd}$$

According to this hypothesis, the enzymes involved in reactions (*a*) and (*b*) would be adaptive and of the hexokinase type. Unadapted strains of *E. coli* can oxidize or ferment pentose phosphate. Consequently, cells adapted to uronate would not be expected to attack free pentose.

Cells which had been adapted to glucuronic acid lost the ability to attack the acid if they were left in an exhausted medium for several hours. Glucose was oxidized immediately when added to such cells. Small amounts of glucose added with glucuronic acid stimulated the utilization of the uronic acid by the cells. This was interpreted to mean that the metabolism of glucose generated compounds which participated in the metabolism of uronate at some stage prior to either oxidation or fermentation. The possibility that glucose stimulated the rate of adaptive enzyme formation was ruled out.

*Polysaccharide.*—Hehre (21) obtained a cell-free extract from *Neisseria perflava* capable of synthesizing a glycogen type of polysaccharide from sucrose without the apparent mediation of glucose-1-phosphate. The same extract synthesized a polysaccharide of the same type with glucose-1-phosphate as the substrate. By exposing thin films of the extract to various gas treatments, some resolution was obtained, and it could be clearly shown that two enzymes were involved. The utilization of sucrose apparently involved transglycosidation of the glucosidic radical from sucrose to a kernel of glycogen-like polysaccharide in the enzyme preparation. This results in the building up of polysaccharide and the splitting off of free fructose units. This enzyme was named amylosucrase. The other enzyme, a phosphorylase, was active upon glucose-1-phosphate but not upon sucrose. By means of the successive action of amylosucrase and $\beta$-amylase, the *in vitro* conversion of the sucrose to maltose was accomplished. The polysaccharide formed by intact cells of *N. perflava* was of the amylopectin-type rather than the glycogen-type produced by the cell extracts. This work and that to be reported below are the first examples of synthesis of glycogen or starch-like polysac-

charides without the intervention of glucose-1-phosphate.

Doudoroff *et al.* (22) and Torriani & Monod (23) demonstrated a polysaccharide-forming enzyme, amylomaltase, in *E. coli.* The former investigators employed a mutant strain which rapidly fermented maltose but did not ferment glucose, while the latter workers used an adaptive enzyme formed in normal cells exposed to maltose. The polysaccharide was synthesized from maltose without the intervention of a phosphorylase since glucose-1-phosphate was not involved. The mechanism of synthesis appeared to be one of transglycosidation as in the cases of the dextran- and levan-forming enzymes of certain bacteria, the sucrose phosphorylase of *Pseudomonas saccharophila* and the amylosucrase referred to above. The reaction may be written as follows:

$$n \text{ maltose} \leftrightarrow n \text{ glucose} + [\text{glucose}]_n. \qquad \text{III}$$

Both groups of investigators have shown the reversible nature of the amylomaltase reaction. If glucose was removed as soon as it was formed in the reaction, the polysaccharide produced was of the starch type (blue complex with iodine). If glucose was permitted to accumulate, no product which formed a blue color with iodine appeared. Since the mutant strain employed by Doudoroff *et al.* (22) did not ferment glucose, it afforded an opportunity to study the direct utilization of maltose. Intact resting cells of this strain fermented maltose with no accumulation of glucose. With dried cell preparations, there was a rapid fermentation of maltose; however, in this case, glucose accumulated in the medium. Considerable evidence was obtained which indicated that maltose was fermented in this strain by way of the transglycosidase to polysaccharide which, in turn, was decomposed by a phosphorylase to glucose-1-phosphate. The latter was fermented according to the usual sequence of glycolytic steps. Such a scheme does not account for the observation that glucose was not produced in the metabolism of maltose by intact cells even though the cells possessed little ability to ferment or oxidize glucose. This paradoxical situation was recognized by the investigators. Although no investigations of the hexokinase activity of this strain were made, they considered the lack of this enzyme to be an unlikely possibility and concluded that some other impairment of the phosphate metabolism of the organism might offer a more probable explanation.

Doudoroff *et al.* (24) studied the phosphorolysis of sucrose by an adaptive enzyme in *Pseudomonas putrefaciens.* The mechanism by which these organisms utilized the glucose moiety of sucrose was investigated, and it was shown that dried cell preparations contained phosphoglucomutase and phosphohexoisomerase. The phosphorylase was of the transglycosidase type discussed previously. This is another example in which there is a more rapid utilization of the disaccharide than of the free hexose. The mechanism remains obscure.

Carlson & Hehre (25) compared the starch synthesized by *C. diphtheriae* from glucose-1-phosphate with several polysaccharides of the starch-glycogen type from animal and plant sources. The major part of the starch from the cells was extracted by the method of Schoch (26) and separated into

two components. One component which resembled corn amylose (27 per cent of total cellular polysaccharide) was obtained in crystalline form. The second fraction (40 per cent of the total cellular polysaccharide) showed mixed properties intermediate between those of amylopectin and amylose of corn starch. A polysaccharide, the properties of which were similar to corn amylose (27), was isolated in crystalline form from *Torula histolytica*.

Radioactive sucrose with $C^{14}$ in either the glucose or the fructose moiety was prepared with sucrose phosphorylase obtained from *P. saccharophila* (28). Radioactive glucose-1-phosphate was obtained by the action of potato phosphorylase on radioactive starch isolated from tobacco leaves which had been exposed to radioactive carbon dioxide during photosynthesis. Incubation of the radioactive glucose-1-phosphate with unlabeled fructose and sucrose in the presence of the phosphorylase gave radioactive sucrose with more than 96 per cent of the radioactivity in the glucose moiety of sucrose. In addition, radioactive fructose obtained by the photosynthetic process using radioactive carbon dioxide and *Canna indica* leaves was equilibrated with unlabeled sucrose and the phosphorylase. The fructose moiety contained over 90 per cent of the activity of the sucrose.

*Carbon dioxide fixation.*—Korkes *et al.* (29) studied the conversion of L-malate to lactate and carbon dioxide by extracts from *Lactobacillus arabinosus* which had been adapted to L-malic acid. The purified extracts required manganous ions and catalytic amounts of diphosphopyridine nucleotide (DPN) for activity. Extracts from unadapted cells were unable to decarboxylate oxaloacetate. Simultaneously with the appearance of activity on L-malate during adaptation, the extracts were able to decarboxylate oxaloacetate to pyruvate and carbon dioxide. The authors believe that two reactions are involved:

$$\text{L-malate} + DPN_{ox.} \rightleftarrows \text{pyruvate} + CO_2 + DPN_{red.} \qquad \text{IV}$$

$$\text{Pyruvate} + DPN_{red.} \rightleftarrows \text{lactate} + DPN_{ox.}. \qquad \text{V}$$

The enzyme which catalyzed reaction IV appeared to be identical with the one previously isolated by Ochoa *et al.* (30) from pigeon liver, except for its DPN specificity. As written, the reaction excludes oxaloacetate (or derivative) as an intermediate. The level of oxidation of the compound involved in the decarboxylation step of reaction IV still remains to be determined.

Plaut & Lardy (31) obtained a 40 to 80 fold purification of oxaloacetate decarboxylase from acetone dried cells of *Azotobacter vinelandii*. Without the addition of $Co^{++}$, $Mn^{++}$, or $Zn^{++}$, the enzyme was inactive. $Mg^{++}$ slightly activated the enzyme while several other ions were inactive. ATP and pyrophosphate were potent inhibitors of the enzyme. Orthophosphate inhibited the reaction in the presence of $Mn^{++}$ presumably by the formation of an inactive phosphate-manganese-enzyme complex. $Co^{++}$ relieved the inhibition by competing with the manganese-phosphate complex for the active center of the enzyme. In contrast to the results obtained with crude preparations from *Micrococcus lysodeikticus* (32) neither the purified nor the crude enzyme from *A. vinelandii* fixed appreciable amounts of carbon dioxide.

The results are similar to those of Utter & Wood (33), who demonstrated with enzyme preparations from pigeon liver that decarboxylation activity was not necessarily accompanied by the ability to fix carbon dioxide. Ochoa *et al.* (30) interpreted such evidence as indicating that fixation did not occur into oxaloacetate, whereas it did into malate. As shown by Wood & Utter (33), however, inactive preparations can be reactivated to bring about carbon dioxide fixation into oxaloacetate. Such evidence indicates that fixation occurs with a $C_3$ compound on the oxidation level of pyruvic acid.

Ajl & Werkman (34) have extended their investigations on the replacement of carbon dioxide as a growth requirement in heterotrophic organisms by compounds of the tricarboxylic acid cycle or their metabolic precursors. By the use of inhibitors presumably specific for certain precursors of either α-ketoglutarate or oxaloacetate, they demonstrated an inhibition of growth of *Aerobacter aerogenes* in the absence of carbon dioxide. Carbon dioxide relieved this inhibition. They believe that compounds which replaced carbon dioxide arose from fixation reactions involving carbon dioxide with either a $C_3$ component or $C_4$ component. The resulting essential $C_4$ and $C_5$ compounds appeared to be oxaloacetate and α-ketoglutarate, respectively. This interpretation negates the possibility of these acids being synthesized by another mechanism, which is hardly in accord with current ideas of comparative metabolism. Monod & Lwoff (35) maintained that their earlier investigations (36) demonstrated that carbon dioxide was required for optimum growth rates, even when the $C_4$ or $C_5$ dicarboxylic acids were present. Under these conditions, yeast extract replaced the carbon dioxide requirement. The active substances in the yeast extract were not identified.

With a cell-free enzyme preparation of *E. coli*, Ajl & Werkman (37) obtained evidence for the reversibility of the following reaction:

$$COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH \xrightarrow{+0.5O_2} COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CO_2. \qquad \text{VI}$$

α-Ketoglutarate was oxidized to succinate in the presence of malonate and sodium bicarbonate containing excess $C^{13}$, until approximately one-half of the substrate was utilized. Malonate inhibited the further oxidation of succinate. The remaining α-ketoglutarate was degraded in order to obtain the α-carboxyl group as carbon dioxide, and the latter was found to contain an excess of $C^{13}$. The methods employed for the separation of α-ketoglutarate, as described by the authors, did not eliminate the possibility of the presence of other labeled keto acids and ether insoluble substances in the α-ketoglutarate fraction which could give rise to labeled carbon dioxide by the degradation procedure employed. Unfortunately, the α-ketoglutarate was not isolated before being degraded. Confirmation of these results would establish another important reaction concerned with carbon dioxide utilization.

*Bacterial photosynthesis.*—In a series of publications, Gest *et al.* (38 to 41) described the interesting phenomenon of photoproduction of hydrogen and nitrogen fixation by *Rhodospirillum rubrum*. During growth in a chemically defined medium, with fumaric, DL-malic or succinic acids as the carbon source

and L-glutamic acid as the nitrogen source, hydrogen and carbon dioxide were produced in the light. Addition of ammonium chloride or some complex nitrogen sources inhibited the production of hydrogen. The photoproduction of hydrogen was not limited to growing cultures of *R. rubrum*. Hydrogen and carbon dioxide were produced when DL-malate and fumarate were added anaerobically to nonproliferating cells exposed to light. Pyruvate under the same conditions allowed only feeble hydrogen production. The combination of glutamate and succinate produced hydrogen, whereas each substance singly did not. Succinate was, however, metabolized photochemically as indicated by the production of carbon dioxide. The evolution of carbon dioxide and hydrogen did not occur in the dark. Quantitatively approximately two moles of carbon dioxide were produced per mole of malate initially present under the conditions described for nonproliferating cells with a helium atmosphere. Approximately the same amount of hydrogen was produced. The hypothesis was presented that the remaining carbon of the malate was assimilated into cellular material at the carbohydrate level, not necessarily by the reduction of carbon dioxide but perhaps by utilization of intermediates of malate metabolism prior to the stage of carbon dioxide. No evidence was given to support this hypothesis. Gest (42) recently demonstrated that considerable amounts of volatile acids are accumulated during the dark decomposition of malic acid. Elucidation of this observation will, without doubt, have many implications in the field of bacterial photosynthesis. Hydrogen production by nonproliferating cells was inhibited by ammonium chloride. Molecular nitrogen also inhibited hydrogen production and with $N_2^{15}$ it was shown that nitrogen was fixed into organic nitrogen as a result of the photochemical reaction. These studies should stimulate investigation of the photochemically induced decomposition of compounds usually encountered in heterotrophic metabolism. Contrary to earlier theories, evidence is accumulating which indicates that these compounds are probably important intermediates in most photosynthetic processes.

## Fat Metabolism

*Fatty acids.*—In a series of six excellent publications, Stadtman & Barker have added greatly to our knowledge concerning the synthesis of fatty acids. The entire investigation was done with the well chosen organism, *Clostridium kluyveri*, whose energy for growth is obtained from the conversion of ethanol and acetate to fatty acids. The first of these publications (43) dealt with the mechanism of synthesis of caproic acid obtained by fermenting unlabeled ethanol and $C^{14}$ carboxyl labeled butyric acid. Degradation of the caproic acid showed conclusively that the $\beta$-position of the acid contained the isotope. Accordingly, the following over-all mechanism was involved:

$$CH_3 \cdot CH_2 \cdot CH_2 \cdot C^*OOH + C_2H_5OH \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot C^*H_2 \cdot CH_2 \cdot COOH + H_2O. \quad \text{VII}$$

The carboxyl carbon of butyric acid condensed with the $\alpha$-carbon of a derivative of ethanol. When $C^{14}$ carboxyl labeled propionic acid and unlabeled ethanol were fermented, propanol, and valeric, heptanoic, acetic, butyric,

and caproic acids were produced. Of the six products, only the products containing an odd number of carbon atoms, propanol, valeric, and heptanoic acids were strongly labeled. The molar specific activities of these compounds indicated that one mole of propionic acid was present in each compound. The mechanism involved for valeric acid was:

$$CH_3 \cdot CH_2 \cdot C^*OOH + C_2H_5OH \rightarrow CH_3 \cdot CH_2 \cdot C^*H_2 \cdot CH_2 \cdot COOH + H_2O \qquad \text{VIII}$$

and for heptanoic acid:

$$CH_3 \cdot CH_2 \cdot C^*H_2 \cdot CH_2 \cdot COOH + C_2H_5OH$$
$$\rightarrow CH_3 \cdot CH_2 \cdot C^*H_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH + H_2O. \qquad \text{IX}$$

The propanol was formed in all probability by direct reduction of propionic acid or a derivative. The fatty acids containing an even number of carbon atoms contained little, if any, radioactivity which showed they were formed from $C_2$ units derived from ethanol.

The remaining publications (44 to 48) reported investigations which attempted to elucidate the mechanism of the multiple condensation of 2-carbon molecules in the biological synthesis of fatty acids. It had been shown previously (49) by means of tracer experiments that the conversion involved an oxidation-reduction process in which ethanol was oxidized to a 2-carbon compound which condensed with acetate to a $C_4$ compound that served as the oxidant and was reduced to butyrate. The series of reactions was postulated as follows:

$$\text{Ethanol} \xrightarrow{-2H} \underset{\text{(acetaldehyde?)}}{\text{Compound a}} \xrightarrow{-2H} \underset{\text{(acetyl P?)}}{\text{Compound b}} \xrightarrow{+\text{ acetate}} \underset{\text{(acetoacetate?)}}{\text{Compound c}}$$
$$\xrightarrow{+2H} \underset{\substack{\text{(crotonate?)} \\ (\beta\text{-hydroxybutyrate?)} \\ \text{(vinyl acetate?)}}}{\text{Compound d}} \xrightarrow{+2H} \text{butyrate} \qquad \text{X}$$

To test these postulated intermediates, a cell-free extract of *C. kluyveri* was prepared which catalyzed the anaerobic conversion of ethanol and acetate to butyric and caproic acids. It was shown that, aerobically, the cell-free extract oxidized ethanol to acetaldehyde (compound a) and that, in the presence of inorganic phosphate, acetaldehyde was oxidized to acetyl phosphate (compound b). Ethanol, in the presence of sufficient inorganic phosphate, was also oxidized to acetyl phosphate. In addition, it was observed that butyrate was oxidized in the presence of inorganic phosphate to acetyl phosphate and acetate. These data pointed to the importance of an active 2-carbon compound in fatty acid synthesis. The reductive conversion of acetyl phosphate and acetate to butyrate by the cell-free extract was convincingly demonstrated with the use of molecular hydrogen as the reductant. Acetyl phosphate was required for the reduction since practically no hydrogen uptake was observed with acetate alone. Apparently a condensation of one mole of acetyl phosphate with one mole of acetate occurred which formed

compound c (at the oxidation level of acetoacetate). The condensation was followed by reduction with hydrogen to butyrate. Synthesis of caproic acid with acetyl phosphate and acetate took place when the amount of acetate was limiting. Acetoacetate was shown not to be the product of the primary condensation of acetyl phosphate and acetate. Acetoacetate was rapidly broken down by a phosphoroclastic split to acetyl phosphate and acetate. By means of isotopes this was shown not to be a reversible reaction. In addition, acetoacetate was reduced by hydrogen to $\beta$-hydroxybutyric instead of butyric acid. Since acetyl phosphate and acetate were reduced to butyric acid and not to $\beta$-hydroxybutyric acid, acetoacetate cannot be a normal intermediate in the process. Of interest, however, was the demonstration of a small amount of $\beta$-keto acid, formed by incubation of the extract with acetyl phosphate and acetate. Identification of this substance was not made. Apparently some 4-carbon intermediate other than acetoacetate was involved in the primary condensation of acetyl phosphate and acetate. An attempt to elucidate the intermediate involved in the second reduction step (compound d) was unsuccessful. $\beta$-Hydroxybutyrate was eliminated as a possibility because it was not reduced to butyrate. Crotonate and isocrotonate were completely inert substrates when added to the cell-free extracts. Vinyl acetate, however, was oxidized in the presence of air to acetyl phosphate and acetate and reduced in the presence of hydrogen to butyrate. These properties of vinyl acetate satisfy the criteria which compound d must have to be an intermediate in the series of reactions. In addition, the rates of oxidation and reduction of vinyl acetate were very satisfactory when compared to the rate of oxidation of butyrate to acetyl phosphate and acetate and to the rate of reduction of acetyl phosphate and acetate to butyrate, respectively.

In spite of this excellent evidence, isotope experiments showed that during the reduction of carboxyl labeled acetate and acetyl phosphate to butyrate in the presence of unlabeled vinyl acetate, the latter did not equilibrate with any isotopic material. Other isotopic experiments designed to demonstrate that vinyl acetate was involved were also negative. These findings emphasize that with respect to evidence for intermediary substances, one may seriously question conclusions based solely on rates of reaction and on quantitative yields. Such evidence may merely indicate a close relationship between a suspected intermediate and the actual intermediate. However, the present case shows that critical tests using tracers or other techniques may reveal the fact that the suspected intermediate and the true intermediate do not show an identical behavior in the metabolic system. A similar situation has been experienced in the reviewer's laboratory (50). An enzyme preparation from pig heart will produce acetylmethylcarbinol from pyruvic acid and acetaldehyde with the apparent formation of $\alpha$-acetolactic acid as an intermediate. A similar preparation from *A. aerogenes* will produce acetylmethylcarbinol from only pyruvate with $\alpha$-acetolactic acid as an intermediate. In the former system added acetaldehyde is an active component, whereas in the latter system, it is apparently inert. The condensation for the formation of $\alpha$-acetolactic acid (5-carbon compound) in both systems pre-

sumably involves pyruvic acid and an unknown 2-carbon compound at the oxidation level of acetaldehyde. The difference in behavior of the two enzyme systems toward synthetic acetaldehyde could account for differences in equilibration with isotopic acetaldehyde. Obviously similar situations may exist in other systems.

Strecker & Wood (51) demonstrated that synthetic acetyl phosphate was not an active intermediate in the phosphoroclastic reaction XI catalyzed by extracts of *E. coli.*

$$CH_3 \cdot CO \cdot COOH + H_3PO_4 \rightleftarrows CH_3 \cdot CO \cdot O \cdot PH_2O_3 + HCOOH. \qquad XI$$

The possibility that biologically produced "acetyl phosphate" might be active was investigated. Such "acetyl phosphate" was prepared by allowing the extracts to dissimilate $C^{14}$ $\alpha$-labeled pyruvic acid to obtain carboxyl-labeled "acetyl phosphate." This material was tested in the presence of non-labeled pyruvic acid for reversal of the above reaction. No reversal was observed as determined by the absence of $C^{14}$ in the $\alpha$-position of the isolated pyruvate.

*Steroids.*—Schatz *et al.* (52), by means of enrichment cultures with cholestrol as the substrate, isolated gram negative, rod shaped bacteria from various soils, manures, compost, peat, and lake sediment. Neither actinomycetes nor molds were isolated. These findings are not in agreement with those of Turfitt (53) who isolated *Nocardia* as the predominant steroid oxidizer in soils. Comparative studies with these isolates and *Nocardia erythropolis* showed that the bacteria attacked a greater variety of steroids.

*Hydrocarbons.*—Saz (54) showed that the $C_{14}$, $C_{16}$, and $C_{18}$ saturated and unsaturated hydrocarbons increased the oxygen uptake of four strains of pathogenic *Mycobacteria.* The increased oxygen uptake was greater than that obtained with glucose or glycerol. No end products were detected and respiratory quotient measurements indicated complete oxidation of the hydrocarbons. It is also possible that dispersing effects by the hydrocarbons on the bacteria may have increased the rate of oxidation of stored materials. Cetyl and stearyl alcohols also increased the oxygen uptake. Hydrocarbons containing 8 carbons inhibited respiration.

## Nitrogen Metabolism

*Amino acids.*—Toennies & Gallant (55) demonstrated the interesting phenomenon of lysis of *Streptococcus faecalis* in suboptimal concentrations of lysine. Initial growth occurred which was proportional to the available lysine. Rapid lysis followed in a medium which contained sufficient phosphate buffer. At low concentrations of phosphate buffer, the phenomenon did not occur. The phenomenon was characteristic of the cells and not of the medium since lysis occurred if cells which had been grown on a low lysine and a high phosphate medium were transferred to a low phosphate medium. If lysis is taken as a criterion of a low internal concentration of lysine, the results of Gale (56) are contradictory. The latter worker found that glycolytic activity aided the escape of free internal lysine from the cells,

whereas in this work, such activity prevented lysis. The lytic phenomenon was discussed in relation to the work of Schoenheimer (57), who had found that lysine was the only amino acid of protein which was not in dynamic equilibrium with the ammonia pool. Lysine, as a consequence, contributed to the stability of the bacterial cell since in its absence lysine was irreversibly lost from bacterial protein, and lysis resulted.

Wood & Gunsalus (58) prepared partially purified serine and threonine deaminases from *E. coli*. As with the toluene-treated suspensions (59) and resting cell suspensions of *E. coli* (60), the purified extract anaerobically deaminated threonine to ammonia and $\alpha$-ketobutyric acid and serine to ammonia and pyruvic acid. Glutathione and adenosine-5-phosphate (AMP) were required to activate the enzyme. Activation by the former appears to involve maintenance of reduced functional sulfhydryl groups. The activation mechanism of AMP is not understood. Lichstein (61, 62, 63) has shown that similar deaminases require both biotin and AMP under some conditions and under other conditions a coenzyme form of biotin in yeast extract. In the experiments of Wood & Gunsalus, yeast extract would not replace AMP as an activator. The possibility remains that the purified extract contained sufficient biotin to react with the AMP to form an active biotin complex different than that present in yeast extract. The role of biotin and AMP in the deaminase reaction requires elucidation. The purified extract did not contain desulfurase activity on cysteine as determined by pyruvate formation. This was in contradiction to the suggestion made by Binkley (64) that enolase, in addition to converting 2-phosphoglyceric acid to phosphopyruvic acid, also catalyzed the deamination of serine and cysteine with the formation of pyruvate. With cysteine, hydrogen sulfide was an additional product. In addition, the cell-free serine deaminase obtained by Binkely from *E. coli* was reactivated by metallic ions, particularly zinc. The purified extract obtained by Wood & Gunsalus contained the deaminases for threonine and serine in the same proportion as did the dried cells. This fact and other properties of the extract suggested that a single entity was responsible for activity on the two substrates. One difference in behavior of the system to serine and threonine was noted which does not necessarily indicate that two enzymes were involved. Serine and threonine were deaminated at the same rate during the first part of the experimental period. Subsequently, the rate on serine fell rapidly while the course of action on threonine continued unchanged. Inactivation of the enzyme only occurred if serine was present during the reaction. Such an inactivated enzyme would not attack threonine. The mechanism of the inactivation of the enzyme is not known.

Hughes (65) demonstrated that cetyltrimethylammonium bromide (cetavlon) accelerated the glutamic acid decarboxylase and glutaminase activity of washed cells or cell extracts of *Clostridium welchii*. The data obtained suggested that a competitive inhibitor of the two enzymes was removed by cetavlon. Bacterial decarboxylases which acted on ornithine, tyrosine, arginine, lysine, and histidine were not accelerated by the addition of cetavlon.

Dawes & Happold (66) partially purified the enzyme complex from *E. coli* known as tryptophanase which catalyzes the oxidation of tryptophane to indole with the consumption of 5 atoms of oxygen. Dialysis destroyed the activity, and reactivation was obtained by the addition of pyridoxal phosphate, riboflavin, and diphosphopyridine nucleotide. Cyanide inhibited the aerobic activity, and verdoperoxidase and cytochrome-*c* stimulated oxygen uptake. An iron containing system is probably involved. Anaerobic fission to indole, pyruvic acid, and ammonia can take place, and the only cofactor required is pyridoxal phosphate.

Stanier & Tsuchida (67) investigated the intermediary metabolism of tryptophane employing the techniques of simultaneous adaptation with an organism belonging to the genus *Pseudomonas*. The organism was isolated by enrichment culture procedures and utilized tryptophane for aerobic growth as its sole source of carbon. The oxidation of tryptophane by the organism was an adaptive process since organisms grown on a medium containing asparagine as the sole source of carbon oxidized tryptophane only after 60 min. of exposure to the amino acid. Cells grown on tryptophane as the sole source of carbon consumed oxygen immediately. The quantity of oxygen consumed was not sufficient to account for the complete oxidation of tryptophane. The oxygen uptake per mole of tryptophane was markedly increased in the presence of 2,4-dinitrophenol (DNP).

Cells which were adapted to L-tryptophane oxidized DL-tryptophane exhibiting a two-step type of oxidation. The first step proceeded at a rate characteristic for the oxidation of the L-isomer and ceased when the total oxygen was half that of an equivalent molarity of the L-isomer. This was followed by a slow continuous increase in the rate of oxygen uptake until the total amount was equivalent to the amount expected by the oxidation of the D-isomer. The secondary rise was intrepreted as adaptive enzyme formation for the D-isomer. Cells adapted to L-tryptophane did not adapt to the D-isomer in the presence of DNP. The specificity exhibited by L-tryptophane adapted cells for the L-isomer was not obtained with D-tryptophane adapted cells. The latter cells were simultaneously adapted to the L-isomer.

The possibility of the oxidation of L-tryptophane by pathways involving indole, serine, indolepyruvic acid, and indoleacetic acid was eliminated by the failure of L-tryptophane adapted cells to oxidize these compounds at rates comparable to that of L-tryptophane. Such cells, however, oxidized kynurenine and kynurenic acid at a rate comparable to the rate of tryptophane oxidation. Cells not adapted to L-tryptophane did not oxidize kynurenine nor kynurenic acid. In spite of the favorable rates of oxidation of these two substances, the amount of oxygen consumed with kynurenine was significantly lower than that obtained with either L-tryptophane or kynurenic acid. It was suggested that kynurenine was oxidized simultaneously by two mechanisms: (*a*) by way of kynurenic acid and (*b*) by an alternate method involving a low oxygen uptake. In the presence of substrate quantities of kynurenine, the second pathway presumably prevails. On the other hand, when kynurenine is present in trace amounts, as it is during tryptophane

metabolism, the pathway via kynurenic acid is preferred.

In view of the results of Stadtman & Barker (48) it seems advisable to interpret cautiously data obtained from rate studies which implicate a compound as an intermediate in metabolic reactions. As described previously, Stadtman & Barker found that vinyl acetic acid satisfied some of the criteria of an intermediate in butyric acid metabolism by cell-free extracts of *C. kluyveri* when judged by rate and quantitative recovery data. However, isotope experiments negated this interpretation.

Lampen *et al.* (68) used a mutant strain of *E. coli* that was unable to synthesize *p*-aminobenzoic acid (PAB) to study the growth responses of the organism due to the addition of purines, pyrimidines, and amino acids in the presence of limiting quantities of PAB. The concentration of PAB required for half maximal growth was considerably reduced by the addition of an amino acid supplement which included methionine. Occasionally, methionine alone reduced the PAB requirement. Addition of a mixture of purines (adenine, guanine, xanthine, hypoxanthine) to the amino acid supplement further reduced the PAB requirement. Purines alone inhibited growth with PAB, and methionine reversed this inhibition. A combination of thymine, the amino acid supplement, and purines effectively replaced PAB. In the latter case, any one of the four purines used in the mixture was effective, and methionine was an essential component of the amino acid mixture. Homocystine could replace methionine, but the final turbidity was approximately one-half of that obtained with methionine. The concentration of a sulfonamide required to produce half maximal inhibition of growth was the same whether methionine or homocysteine was present in this medium. Therefore, the main action of PAB did not appear to be in the methylation of homocysteine but in some reaction intermediate between cystine and serine (in the amino acid supplement) and homocysteine. These results are in contrast to the report of Winkler & de Haan (69) that homocysteine with or without choline did not replace methionine as a sulfonamide antagonist. This discrepancy obviously requires further investigation. Growth of the mutant with PAB, amino acid supplement, and purines was inhibited by the addition of sulfonamides. Pteroylglutamic acid did not possess any demonstrable antisulfonamide activity. When amino acids, purines, and thymine were present, the concentration of sulfonamide required for inhibition was many times greater. In view of the requirement of thymine and purine for growth, it is surprising that pteroylglutamic acid (PGA) did not replace PAB in this mutant strain. During growth of the parent and the mutant strain on optimal concentrations of PAB, a material is synthesized which is able to support maximal growth of *S. faecalis R* on a medium free of pteroylglutamic acid. Apparently some compound similar to pteroylglutamic acid is synthesized which is not necessarily identical to it and may be concerned with purine and thymine synthesis.

Holland & Meinke (70) found that PGA was effective in promoting the synthesis of serine by *S. faecalis R*. In view of the results of Lampen *et al.* (71) and those cited immediately above, these results are in harmony with

the view that PAB or some derived form (PGA) is involved in the cysteine →cystathionine→homocysteine series of reactions.

Hift & Wallace (72) found with *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* that homocysteine would substitute for cystine if serine was also supplied. Methionine was not required for maximal growth nor would it serve as a cystine precursor. These results suggested that these organisms did not transform methionine into homocystine. The coupling of serine with homocystine apparently took place to form cystine presumably via cystathionine.

Broquist & Snell (73) studied the mechanism of histidine synthesis in lactic acid bacteria and the possibility that histidine was a precursor of purine synthesis. The histidine requirement of *S. faecalis*, which required either PGA or a mixture of thymine and purine bases for growth, was compared in a medium which contained PGA but no purine bases and in a medium which contained thymine and hypoxanthine in place of PGA. The histidine requirement was identical in both cases. Therefore, histidine did not serve as a precursor in purine synthesis. *Lactobacillus casei* was used to test the hypothesis that purines were precursors in the synthesis of histidine. This organism was trained to grow well without added histidine. In the absence of PGA and an excess of thymine, it responded to graded levels of purines. Under conditions in which histidine had to be synthesized by the organism, greater amounts of guanine were required for maximum growth than under condtions not calling upon the organism to synthesize as much histidine. This greater demand for purine was indicative of its need for histidine synthesis. Imidazolealdehyde inhibited the growth of *L. arabinosus*. The inhibition was partially overcome by the individual addition of either purine bases or histidine and was completely eliminated by a mixture of the two. These data are consistent with the view that in this organism purine bases serve as precursors for histidine synthesis. Imidazolepyruvic acid replaced histidine for both *L. arabinosus* and *S. faecalis* when vitamin $B_6$ was present, but not when the vitamin was absent. Apparently vitamin $B_6$ is involved in the conversion of the keto acid to histidine, probably by transamination.

McLean & Fisher (74) further elucidated the observation that *Serratia marcescens* consumed oxygen more rapidly during an active period of growth than when cells were in a resting condition. In a medium which contained all the essential ingredients for growth except a nitrogen source, with citrate or glycerol as the sole source of carbon, there was a definite resting cell rate of oxygen uptake which increased when ammonium chloride was added as the sole source of nitrogen. The effect was studied in greater detail with other sources of carbon and nitrogen. With a mixture of glycerol and citrate as a carbon source, asparagine, urea, and DL-alanine served as nitrogen sources, while several other amino acids were inactive. Ammonium chloride did not serve as an adequate nitrogen source when the carbon sources were lactate, acetate, succinate, or ethyl alcohol. The quantity of oxygen associated with the assimilation of known quantities of each nitrogen source was determined.

It varied over a three-fold range depending upon the temperature and nature of both the nitrogen and carbon sources. The quantity of oxygen was also modified by adding sulfathiazole.

The incorporation of $C^{14}$ carboxyl labeled glycine into cellular protein of *Torula utilis* was demonstrated by Friedberg & Webb (75). Glucose added to the medium increased the incorporation; aerobic conditions favored incorporation and cyanide totally inhibited it.

Maas & Davis (76) found that D-serine in small concentrations inhibits the multiplication of *E. coli.* The inhibition was effective after a few normal divisions took place at normal rate. The duration of the inhibition depended upon the concentration of the D-serine. Antagonism to the inhibition was provided by DL-alanine and glycine.

Rogers (77) studied the breakdown of N-acetylglucosamine by cell suspensions of *Streptococcus hemolyticus* and other streptococci. The products of the breakdown were 1 mole of ammonia, 1.2 to 1.7 moles of lactate, and 1 equivalent of volatile acid per mole of N-acetylglucosamine utilized. Inorganic phosphate appeared to be required for the reaction since in the absence of phosphate buffer, the reaction was considerably slower. Attempts to resolve the process of degradation into a sequence of deacetylation, deamination, and subsequent utilization of the glucose residue failed. The three processes had equal rates. The utilization of N-acetylglucosamine was strongly inhibited by glucose. The most likely mechanism for the reaction appeared to be the breakdown of the glucopyranose structure with the subsequent deamination of an intermediate amino compound.

Updegraff (78) found bacteria capable of producing phenol and *p*-cresol widely distributed in marine sediments from the continental shelf of southern California and from the intertidal zone of Louisiana. The organisms obtained from enrichment cultures with sea water containing L-tyrosine, casein, or peptone produced phenol or *p*-cresol. Attempts to isolate pure cultures of *p*-cresol producing organisms were unsuccessful, but 10 pure cultures of phenol-producing bacteria were isolated from marine sediment samples and one from oil well cellar water. The mechanism of phenol production by this organism apparently differs from that operative in *E. coli* in that *p*-hydroxybenzoic acid is not an intermediate.

*Nitrogen fixation.*—Rosenblum & Wilson (79) examined the ability of 15 species of the genus *Clostridium* to fix molecular nitrogen. All but three, *C. sporogenes*, *C. perfringens*, and *C. acidiurici* were capable of fixing nitrogen as indicated by tracer studies with $N_2^{15}$. Lindstrom *et al.* (80) with the aid of $N_2^{15}$ showed that *Rhodospirillum rubrum* fixed elemental nitrogen and that fixation is associated with photoreduction [See Gest *et al.* (39]. An attempt to correlate the hydrogenase activity with nitrogen fixation is being made. Species of two genera of the family Thiorhodaceae which possessed hydrogenase were found to fix molecular nitrogen.

In a series of carefully controlled experiments Segal & Wilson (11) demonstrated that hydroxylamine is not utilized as a source of nitrogen by *Azotobacter vinelandii* even in nontoxic concentrations of 1 to 2 $\mu$g. per ml.

Delayed growth on toxic quantities of hydroxylamine was presumably due to the slow decomposition of the amine to ammonia, the latter serving as a nitrogen source.

*Purines*.—Karlsson & Barker (81), with the aid of $C^{14}$, have studied the mechanism of decomposition of uric acid and synthesis of acetic acid by *Clostridium acidiurici*. This organism anaerobically decomposes uric acid, hypoxanthine, guanine, and xanthine to ammonia, acetic acid, and carbon dioxide. The following equation represents the decomposition of hypoxanthine:

$$\text{Hypoxanthine} + 6.5\ H_2O = 4\ HN_3 + 1.25\ CH_3COOH + 2.5\ CO_2. \qquad \text{XII}$$

The high yield of acetic acid is significant since the direct decomposition of the $C_3$ chain in 1 mole of hypoxanthine cannot give more than one mole of a $C_2$ compound. Condensations of carbon derivatives must occur. Glycine may be involved in the decomposition of the purine since it was decomposed when added in the presence of purine. Labeled uric acid in the presence of excess unlabeled glycine and carbon dioxide was fermented and the isotope content of the acetic acid and carbon dioxide determined. Unlabeled uric acid was also fermented in the presence of labeled glycine and carbon dioxide and the isotopic content of the acetic acid and carbon dioxide determined. A summary of the results, as presented by the authors, is given in Table I. From this

TABLE I

THE ISOTOPE CONTENT OF ACETIC ACID AND CARBON DIOXIDE FROM URATE

| Source of Radioactivity | Percentage of Radioactivity Derived from Each Source | | |
|---|---|---|---|
| | $HAc\text{–}CH_3$ | HAc–COOH | $CO_2$ |
| Urate, 2 and 8 positions | 45 | 2 | 52 |
| Urate, 4 position | 0 | 0 | 44 |
| Urate, 5 position | 18 | 82 | 0 |
| Urate, 6 position | 0 | 0 | 3 |
| Glycine, methylene group | 8 | 25 | 0 |
| Glycine, carboxyl group | ~1 | ~1 | 7 |
| Carbon dioxide | 17 | 17 | |
| Total | 89 | 127 | 106 |

table it is evident that the ureide carbons of uric acid, i.e., carbons 2 and 8 contribute almost half of the methyl carbon of acetic acid. Position 5 of uric acid and the methylene carbon of glycine contributed heavily to the carboxyl of acetate. Carbon dioxide contributed its carbon evenly to the methyl and carboxyl group of acetic acid. Adequate space is not available to discuss the implications of this important work; for that reason, the reader is referred to the original article. These results to some degree are comparable with the results of Sonne *et al.* (82) who showed that formate was a precursor of the ureide carbons (2 and 8) of the uric acid excreted by pigeons. The car-

boxyl carbon and the $\alpha$-carbon of glycine served as precursors for carbons 4 and 5 respectively in the uric acid.

In addition, Sakami (83) has recently demonstrated that formate is a precursor for the $\beta$-carbon of serine when administered with glycine to rats. In the bacterial system described above, carbons 2 or 8 of uric acid may condense with carbons 5 and 4 (methylene and carboxyl carbons of glycine derivatives, respectively) to form a sequence of carbon atoms such as exists in serine. Subsequent oxidations and reductions of this 3-carbon skeleton could account for the methyl group of acetic acid arising from the 2 or 8 position of uric acid, the carboxyl carbon arising from carbon 5 and carbon dioxide arising from carbon 4. As the authors state, the mechanisms involved in the overall transformations are complex and elucidation can probably best be achieved by enzymatic methods.

*Nucleic acids.*—Abrams *et al.* (84) employed $N^{15}$ as a tracer to observe the formation of protein and nucleic acid purines in *Torulopsis utilis.* It was hoped to obtain a further insight into the view of Caspersson (85) that ribonucleic acids are intimately associated with protein synthesis. Yeasts which had a high nitrogen content were obtained by cultivating the yeast with excess ammonia. High nitrogen containing yeast began to divide immediately upon the addition of a carbon source. Cell suspensions of the yeast were exposed to ammonium sulfate solutions which contained an excess of $N^{15}$. They were removed from the suspending medium, and by fractionation procedures, nucleic acid purines and cellular proteins were obtained. There was no detectable fixation of $N^{15}$ in the nucleic acid purines, a trace in the protein, and a small amount in the total yeast. It was concluded that no net assimilation of nitrogen occurred, and synthesis of protein and nucleic acid purines took place to a negligible extent.

Similar experiments were conducted with yeast with a low nitrogen content obtained by cultivating the yeast with minimal amounts of ammonia. Cell suspensions of this type of yeast very rapidly assimilated ammonia nitrogen containing an excess of $N^{15}$, but multiplication did not occur. From 95 to 99 per cent of the ammonia disappeared from the medium. The isotope content of the remaining ammonia did not change which indicated that potential sources of ammonia in the cell were not in equilibrium with the exogenous ammonia. Despite the great assimilation of nitrogen, there was no total increase in nucleic acid purine; in fact, the ratio of purine nitrogen to total nitrogen was markedly reduced after nitrogen assimilation. There was, however, a rapid turnover of nucleic acid purine as indicated by the high $N^{15}$ content. It appeared from these results that whatever role nucleic acids play in the synthesis of proteins from ammonia, the rate of turnover of nucleic acids (as determined from the $N^{15}$ content in the purines) was a greater factor than the increase in concentration of nucleic acids. The rate of turnover was further studied by exposing high nitrogen containing *Torulopsis*, which had incorporated $N^{15}$, to a medium which contained ordinary ammonium sulfate. The extent of dilution by the nonisotopic nitrogen was determined in the cellular protein and nucleic acid purine. The dilution

of $N^{15}$ in the cellular protein was only a fraction of that expected from the amount of growth which had taken place during the period the $N^{15}$ yeast was exposed to the nonisotopic ammonia. This indicated, as in the previous experiments, that the protein nitrogen was not in equilibrium with the exogenous ammonia. Considerable dilution of the glutamate and aspartate obtained from the protein hydrolysates had taken place. This suggested the importance of these two acids in the primary fixation of ammonia. The nucleic acid purine and the arginine from the protein hydrolysate remained unchanged. The nitrogen precursors of the nucleic acid, purine, and arginine must have been preformed during growth in $N^{15}$ since they were not significantly diluted during the period of growth in ordinary nitrogen.

Of interest in these experiments is the apparent lack of equilibrium of the ammonia pool with protein nitrogen in yeast as compared to mammalian tissues where a complete dynamic equilibrium exists. It cannot be stated at this time whether this is caused by irreversible fixation of ammonia or by a low permeability of the cell wall of yeast for ammonia.

The synthesis of nucleic acids in cultures of *E. coli* was studied by Morse & Carter (86). Ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) concentrations were determined as a function of the number of cells. The quantity of RNA per cell increased during the lag phase of growth and reached a maximum when actual cell multiplication began. The amount of RNA per cell declined during multiplication. Similar changes which were significantly smaller occurred in the DNA concentration. By the use of radioactive phosphorus as an indicator of nucleic acid content, the rapid synthesis of RNA was confirmed. In the strain B/r, an ultraviolet-resistant strain, the amount of DNA per cell was 3 to 4 times that found in the parent strain.

Stephenson & Moyle (87) studied the phosphorus distribution in *E. coli*. Washed suspensions of the organisms grown on a tryptic digest of casein with glucose contained acid-insoluble phosphorus to the extent of 15 to 16 μg. per mg. cells and acid-soluble phosphorus of 1 μg. per mg. cells. On incubating the cell suspension in bicarbonate buffer and in the presence of lactate, the acid-insoluble fraction decreased approximately 50 per cent. This phosphorus appeared in the supernatant as 60 per cent organic and 40 per cent inorganic. The total nucleic acid content of freshly harvested cells was approximately 15 per cent of which about 70 per cent was RNA and 30 per cent was DNA. After incubation with lactate, the RNA decreased by 60 per cent and the DNA by 20 to 33 per cent. If the cells were incubated with glucose instead of lactate, there was no degradation of nucleic acids.

Schade (88) found that cobalt in concentrations of 1 to 100 p.p.m. was inhibitory to the growth of a large number of organisms representing many genera. Anaerobic, aerobic, gram positive, and gram negative species were among those affected. The quantity of cobalt required to inhibit depended upon the particular species and upon the composition of the medium. Histidine completely reversed the growth inhibition when the molar ratio of histidine to cobalt was 2:1. The effect of cobalt on nucleic acid metabolism is cited immediately below.

Levy *et al.* (89) studied the effect of cobalt on the relationships between nucleic acid concentration and growth rate in *Proteus vulgaris.* In heavily inoculated media in the absence of cobalt, there was a rapid increase of growth as determined by turbidimetric and dry weight measurements. The amount of DNA of the cells fluctuated somewhat but in general remained at a more or less constant value. The amount of the RNA of the cells, on the other hand, began to increase almost immediately and reached a maximum of approximately 2.5 times that of its initial concentration. This was followed by a gradual decline. In a similar experiment to which cobalt was added and in which no growth occurred, the concentration of the DNA showed the same variation as did controls without cobalt. The concentration of the RNA in the presence of cobalt, likewise, followed the same pattern as did the controls without cobalt. Dry weight measurements indicated no growth had occurred. In the absence of cobalt, rapid synthetic activity and growth were associated with a marked increase of RNA of the cell. This same increase was noted when the synthetic activity was stopped by the addition of cobalt. The authors suggest that the effect of cobalt might be attributed to an effect on the process by which RNA leads to protein synthesis.

The same investigators (90) studied the effect of cobalt on the phosphorus turnover rate in the nucleic acids of *Proteus vulgaris.* Cells which had radioactive phosphorus in their RNA and DNA were obtained by growing the organisms in a medium containing radioactive phosphorus. These organisms served as a heavy inoculum for two media, one containing cobalt. After incubation, the cells were removed and the specific radioactivity of the RNA and DNA phosphorus was determined. There was a decrease in the specific activity during the incubation period in both the presence and absence of cobalt. However, in the presence of cobalt, the decrease was much greater with the greatest decrease in the specific activity of the DNA. Turnover rates of the nucleic acids were calculated and found to be 12.2 hr. for the RNA of the control and 7.5 hr. for the cobalt-treated cells. For the DNA, the turnover rates were 32.9 hr. and 5.9 hr for the control and cobalt-treated cells, respectively.

Chargaff & Saidell (91) isolated a purified nucleoprotein from *Mycobacterium tuberculosis* var. *avian.* Considerable purification was obtained; however, the most purified preparation contained mixtures of RNA (13 per cent) and DNA (87 per cent). Attempts to demonstrate that the protein moiety possessed basic properties of the protamine or histone type were not successful. Cleavage of the nucleic acid-protein bond was accomplished with the action of saturated sodium chloride solution or by the detergent action of sodium desoxycholate. The nucleic acids from such solutions were purified by electrophoresis; however, complete separation of RNA from DNA was not indicated.

In a second publication from Chargaff's laboratory (92), the composition of the DNA obtained by purification of the nucleic acid preparations from *M. tuberculosis* discussed above was determined by the micro method of paper chromatography developed in the same laboratory (93). The percent-

ages of the several purines and pyrimidines in the DNA of the tubercle bacillus and of yeast are given in Table II.

TABLE II

PURINE AND PYRIMIDINE CONTENT OF DNA

| | Adenine | Guanine | Cytosine | Thymine |
|---|---|---|---|---|
| *M. tuberculosis* | 3.9 | 10.1 | 6.8 | 3.2 |
| Yeast | 8.5 | 5.5 | 3.9 | 8.3 |

It is evident from the above table that the two microbial desoxyribonucleic acids differ appreciably from each other. The absence of 5-methylcytosine which had previously been reported by Johnson & Coghill (94) to be characteristic of tubercle bacilli is of interest. In this work, a special effort was made to detect this pyrimidine, but no evidence could be found for its existence. Approximately 25 per cent of the nucleic acid nitrogen was not accounted for, and as a result, some major constituents of these nucleic acids probably have not been identified. The sugar moiety from the purine nucleotides of the bacillary desoxynucleic acid had properties similar to the sugar obtained from thymus nucleic acid which presumably is 2-desoxyribose.

Zamenhof & Chargaff (95) prepared a cell-free desoxyribonuclease from yeast. When freshly isolated from the yeast, it did not depolymerize DNA but became active upon storage. The inactivity was due to an inhibitor in the preparation which was slowly destroyed during storage by a yeast protease. The three components, desoxyribonuclease, inhibitor, and protease, were partially resolved, and some of their properties are discussed. The inhibitor is specific for yeast desoxyribonuclease as it does not affect the desoxyribonuclease from other sources. The authors have speculated on the biological implications of these substances as agents in controlling the state of polymerization of DNA.

In order to obtain isotopically labeled nucleic acids for the study of nucleic acid metabolism and a source of labeled nucleotides and nucleosides, DiCarlo *et al.* (96) propagated *Saccharomyces cerevisiae* on a medium containing excess $N^{15}$ in the form of ammonium sulfate. The isotopically labeled nucleic acid was isolated from the cell mass, and it was shown that the isotopic nitrogen was uniformly distributed throughout the purine and pyrimidines.

## EFFECT OF ANTIBACTERIALS ON INTERMEDIARY METABOLISM

*Streptomycin*.—In their previous work, Henry *et al.* (97) demonstrated an inhibitory action of streptomycin on the oxidative metabolism of resting cells of some bacteria on certain carbohydrates and carbohydrate intermediates. They have now extended this work to multiplying bacteria (98). Studies with *Shigella sonnei* showed that coincident with inhibition of multiplication by streptomycin, there was an inhibition of oxygen uptake. The overall inhibition of oxygen utilization paralleled the inhibition of substrate (glucose or pyruvate) utilization. In the presence of greater amounts

of streptomycin, larger amounts of acetic acid accumulated. Under conditions where multiplication was prevented by streptomycin, the nitrogen content of *Bacillus cereus* was lower than in controls. The presence or absence of glucose did not materially change the nitrogen content with or without streptomycin. The phosphorus content of multiplying cells in the absence of glucose remained constant, but in the presence of glucose, it decreased. The presence of streptomycin further decreased the cellular phosphorus and increased the amount of reducing substance in the cells. In view of our present lack of knowledge of the mode of action of the antibiotic, the authors found it difficult to interpret the changes in nitrogen, phosphorus, and reducing values due to streptomycin. There was, however, a definite effect on the oxidative metabolism of carbohydrate by streptomycin. Whether these changes represent the primary action of streptomycin cannot be stated.

In a series of publications, Umbreit and his colleagues reported an inhibitory action of streptomycin on the respiration of *E. coli*. These results were interpreted on the basis of a specific inhibition of the pyruvate-oxaloacetate condensation reaction of the tricarboxylic acid cycle. In the first of these publications (99), they confirmed the work of Geiger (100) which showed that following the oxidation of fumarate by a particular strain of *E. coli*, there was a marked stimulation of serine oxidation. Streptomycin, if present during the oxidation of fumarate, inhibited this stimulated oxidation of serine. The same effects were obtained with threonine and with mixtures of fumarate and pyruvate. The authors concluded that streptomycin interfered with the formation of some intermediate which was required for the complete oxidation of serine, threonine, or pyruvate. This substance presumably was oxaloacetate. This work has been extended in a second publication (101). It was found that fresh cells of *E. coli* possessed an active oxaloacetate decarboxylase which became less active as the cells were stored in the cold. With the older cells, pyruvate was oxidized probably to acetate, and streptomycin had little effect on this oxidation. When both oxaloacetate and pyruvate were present as substrates there was a short lag period with little oxygen uptake followed by a rapid and linear rate of oxygen consumption in excess of that obtained with pyruvate alone. Streptomycin markedly inhibited this latter oxidation. Oxaloacetate as the sole substrate was slowly oxidized. The interpretation of these data was that (*a*) oxaloacetate and pyruvate were condensed, and the condensation product oxidized, and (*b*) in the presence of streptomycin, which inhibited the condensation reaction, the substrates were oxidized to acetate. In view of the complexity of the normal metabolism of pyruvate by *E. coli* and the lack of quantitative data concerning the products produced from pyruvate and oxaloacetate under the conditions used by these workers, it is premature to speculate on the mechanisms involved. Several investigations have shown that cells of *E. coli* grown under very similar conditions possess very active enzymes capable of performing the dismutation reaction (XIII) and the phosphoroclastic reaction (XIV). These reactions occur both aerobically and anaerobically.

$$2\ CH_3 \cdot CO \cdot COOH + H_2O \rightarrow CH_3 \cdot CHOH \cdot COOH + CH_3COOH + CO_2 \quad \text{XIII}$$

$$CH_3 \cdot CO \cdot COOH + H_3PO_4 \rightarrow CH_3 \cdot CO \cdot O \cdot PH_2O_3 + HCOOH \quad \text{XIV}$$

Reactions of this sort may be influenced by the action of streptomycin and as a consequence alter the observed oxidative metabolism as measured by oxygen uptake.

The metabolic properties of strains of *E. coli* resistant to or dependent on streptomycin were also studied (102). Using techniques similar to those described above for sensitive strains, the conclusion was reached that the resistant and dependent variants do not possess the ability to carry out the oxaloacetate-pyruvate condensation in detectable amount.

In another publication (103), the effect of streptomycin on the condensation reaction in animal tissue was investigated. With homogenates prepared with isotonic solutions, streptomycin had no effect on the oxygen-uptake with mixtures of pyruvate and oxaloacetate. In addition, homogenates were prepared in water and fortified with cofactors. Such homogenates contain altered mitochondria. The oxidation of mixtures of pyruvate and oxaloacetate by these homogenates was inhibited by streptomycin. Preincubation of the saline homogenate with streptomycin resulted in the same inhibition of oxidation as observed with the water homogenate. This suggests that permeability is important here.

These extensive investigations have demonstrated the inhibitory effect of streptomycin on a respiratory process both in the bacterial and mammalian cell. An objection may be raised to the claim that streptomycin specifically inhibited the oxaloacetate-pyruvate condensation reaction in bacteria. A great deal of work, both published and unpublished, has been done in an attempt to demonstrate the existence in bacteria of a cycle similar to the tricarboxylic acid cycle. Qualitative evidence suggests that the cycle does occur, but when the techniques of quantitative biochemistry have been applied, the results have been negative. Very recently Novelli & Lipmann (104) and Stern & Ochoa (105) have shown that the condensation of an acetyl derivative and oxaloacetate with the formation of citrate occurs in *E. coli*. The conversion of citrate to succinate through isocitrate, oxalosuccinate, and $\alpha$-ketoglutarate in bacteria has not been demonstrated as an orderly sequence of events. Until such reactions have been demonstrated, it would appear unwise to make the assumption that they do occur regardless of the excellent qualitative evidence which exists.

*Penicillin.*—Hotchkiss (106) studied the effect of penicillin G on the assimilation of amino acids by respiring staphylococci. Previously he (107) had shown that such cells rapidly take up amino acids and couple them into cellular protein. In the presence of penicillin, the amino acids disappear at the same rate; however, the nitrogen compounds produced are recovered in the suspending medium. These compounds were not characterized. When uracil was present with the amino acids, there was a faster rate of disappearance of the uracil in the presence of penicillin than in its absence. The uracil utilized could not be recovered from the cells.

In this connection, the work of Park & Johnson (108) is of interest. They showed that cells of *Staphylococcus aureus* grown in broth containing 0.1 unit penicillin per ml. accumulate a labile phosphate compound which was extractable with trichloroacetic acid. The compound was present in greater quantity than in cells grown in the absence of penicillin. Purified fractions of the compound were obtained by alcohol precipitation of the barium salt. The material absorbed strongly at 262 m$\mu$. After hydrolysis with 25 per cent sulfuric acid, a colorimetric test for uracil and cytosine was obtained. Over half of the light adsorption was caused by uracil. However, the possibility was recognized that during such acid hydrolysis, cytosine was converted to uracil. The light absorption of the unhydrolyzed material did not correspond to cytosine. A reducing group was released after acid hydrolysis for 3 min.; 0.2 mole pentose was present per mole of labile phosphate as judged by the orcinol method, but longer periods of heating increased the apparent pentose content. The quantities of this material produced in the presence of penicillin varied from day to day.

In previous publications Gros & Macheboeuf (109, 110) found that penicillin inhibited the degradation of the ribonucleic acid of autolyzing cells of *Clostridium sporogenes*. Crystalline pancreatic ribonuclease was not inhibited, neither was the phosphatase of *C. sporogenes* acting on glycerophosphate. However, penicillin did inhibit the degradation of ATP, adenylic and guanylic acids by resting cells of this organism. In their latest publication (111), they have tested an intestinal nucleotidase preparation acting on adenylic acid for inhibition with varying amounts of penicillin. No inhibition of the appearance of inorganic phosphate was observed. Similarly, no effect was found on the phosphatase of *Staphylococcus albus* acting on various mononucleotides. They, therefore, suggested that penicillin acts on the nucleotide-degrading system of *C. sporogenes* other than phosphatase. In the reviewer's laboratory (112), an anaerobic degradation of guanylic acid by resting cells of three strains of *E. coli* (penicillin-insensitive) was studied. The breakdown of adenylic and guanylic acid by resting cells of *S. aureus* (penicillin-sensitive) was also examined. Penicillin was found to have no effect on the metabolism of the nucleotides by all the organisms tested, as measured by the disappearance of pentose from the medium. Aerobically, penicillin had no effect on the degradation of adenylic acid, guanylic acid, or ribose-5-phosphate by resting cells of *S. aureus*. The results of Gros & Macheboeuf were not confirmed with these organisms.

Gale & Rodwell (113) continued their studies on the assimilation of amino acids by bacteria, with special regard to the nature of resistance to penicillin developed in *S. aureus*. Organisms which exhibited increased resistance to penicillin required greater amounts of penicillin to prevent the passage of glutamic acid into the cell. As the organism became highly resistant to penicillin, they acquired greater synthetic abilities as judged by the ability to grow on less complex media. Organisms which were made non-exacting in nutritional requirements by serial transfers in amino acid-free medium became penicillin resistant. The primary action of penicillin was

suggested to be an inhibition of ribonucleic acid synthesis which is essential for the amino acid assimilation process to occur. The inhibitory action of pencillin on ribonucleic acid synthesis had previously been reported (114, 115).

Hunter & Baker (116) and Grelet (117) demonstrated that penicillin inhibited the growth of *Bacillus subtilis* in media containing inorganic nitrogen as the only source for growth. This suggests that the primary mode of action of penicillin is not necessarily concerned with the incorporation of preformed amino acids [cf. Gale (118)].

Pratt & Dufrenoy (119) employing their method of double staining of penicillin assay plates with triphenyltetrazolium chloride and with trypan blue obtained results which were interpreted as evidence that penicillin inhibited the enzyme systems involved in hydrogen transfer and in the dephosphorylation of complex ribonucleates in gram positive cells.

Maass & Johnson (120) demonstrated that in *Staphylococcus* cells equilibrated with equal volumes of penicillin G solutions of varying concentrations, two types of uptake of penicillin occurred: (*a*) a specific uptake of 0.8 unit per ml. of cells that was independent of the intracellular penicillin concentration and (*b*) a diffusion of penicillin into the cell so that the intracellular water had the same penicillin concentration as the extracellular water. The specific uptake was independent of time of equilibration. Penicillin containing radioactive sulfur $S^{35}$ was used to demonstrate the firmness with which the penicillin was specifically bound. Extensive washing or equilibration with solutions of nonradioactive penicillin did not release the bound radioactive penicillin from the bacterial cells. Calculations showed that 750 molecules of penicillin per bacterial cell were specifically absorbed. Yeast cells did not absorb penicillin nor did penicillin penetrate the cell wall. Cooper & Rowley (121) obtained similar results with the aid of radioactive penicillin. They also found that under conditions of rapid growth more penicillin was bound per cell.

*Sulfonamides.*—Sevag & Steers (122, 123) have continued the studies on the mechanism of resistance to the sulfonamides. Associated with the development of resistance was the partial or complete loss of ability to synthesize amino acids. In most cases, this defect was compensated by metabolizing glucose. Since the syntheses of most amino acids were dependent on glucose metabolism and since some of these syntheses were susceptible to sulfathiazole, it was the authors' view that the enzymes involved in the metabolism of glucose and the tricarboxylic acid cycle represented the critical sites of sulfonamide action. They also presented evidence that resistant and susceptible cells had separate alternate metabolic pathways which involved glucose and tryptophane.

Gots & Sevag (124) showed that the inhibition by sulfathiazole of the aerobic respiration in *Diplococcus pneumoniae* was antagonized by methylene blue. Inhibition of methylene blue reduction by dehydrogenases was obtained if the sulfonamide reacted with the cells in the absence of methylene blue. Riboflavin, under certain conditions, also antagonized the inhibition of

sulfathiazole. These results were interpreted to mean that sulfathiazole and methylene blue can displace riboflavin from the enzyme as the reaction proceeds.

*Cofactors in metabolism.*—Novelli *et al.* (125) demonstrated that coenzyme A and the products of its degradation with liver extracts stimulated the growth of *Acetobacter suboxydans* above that attributable to their pantothenic acid contents. The stimulatory effects were destroyed by intestinal phosphatase. These and other properties indicated that the factor from heart-muscle reported by King *et al.* (126) to have similar stimulatory properties could be coenzyme A or one of its degradation products. Cohen *et al.* (127) demonstrated the synthesis of coenzyme A from pantothenate and glutamate by washed suspensions of *Clostridium saccharobutyricum* in the presence of glucose. No synthesis was observed when other amino acids were substituted for glutamate. Pantoyltaurine inhibited the synthesis.

Lichstein (63) (discussed briefly above) presented evidence that a coenzyme form of biotin was required for the aspartic acid deaminase activity in *P. vulgaris* and *E. coli*. The preparations used were not activated by biotin and adenylic acid but were activated by yeast extract. The activity of yeast extract was approximately 100 times greater than could be accounted for by its assayable biotin content. In a later publication (128) the same authors were able to separate from yeast extract a substance (or substances) by paper strip chromatography which activated the deaminases of aspartic acid, serine, and threonine. The material was not adenylic acid or biotin as such, but it did contain bound biotin as determined by microbiological assay.

Billen & Lichstein (129) studied an aspartic acid decarboxylase contained in cell suspensions of *Rhizobium trifoli*. The end product of the decarboxylation was $\beta$-alanine, which was determined by microbiological assay using *Saccharomyces fragilis*. Cells harvested during the logarithmic growth phase showed the most active decarboxylation. The optimum temperature for activity was 46°C. and the optimum pH was between pH 5.2 and 6.2. Comparative studies disclosed that the enzyme was also present in *E. coli* but absent in several other organisms.

Melville *et al.* (130) synthesized biotin with $C^{14}$ in the ureido carbon atom in order to test the hypothesis that the vitamin might enter into biological carbon dioxide transferring mechanisms by virtue of an opening and closing of the ureido ring system. *L. arabinosus* was grown under conditions where it required biotin and fixed carbon dioxide. Using this test system, it was found that there was no replacement of the $C^{14}$ of the radiobiotin with non-radioactive carbon. It is evident that if biotin was required for carbon dioxide fixation, it did not involve a transfer of the ureido carbonyl group of the biotin molecule.

Silverman (131) showed that atabrine stimulated the carboxylase activity of an apoenzyme preparation obtained from *Torula utilis* when fortified with cocarboxylase and manganese ions. It was found that the apoenzyme contained an active phosphatase. Thiamine was about 10 times more effective than atabrine as an inhibitor of the phosphatase activity. The conclusion

was reached that the inhibition by atabrine of the dephosphorylation of cocarboxylase does not account for the growth inhibitory effects of the drug.

Velluz *et al.* (132) tested thiamine triphosphate for cocarboxylase activity with an apoenzyme prepared from baker's yeast. Thiamine triphosphate, it is believed, bears the same relationship to thiamine diphosphate (cocarboxylase) as adenosinetriphosphate does to adenosinediphosphate. The authors reported that thiamine triphosphate had more active physiological properties in the heart than either thiamine or cocarboxylase. However, in the yeast carboxylase system, it was found that thiamine triphosphate showed only 80 per cent as much activity as cocarboxylase.

Using *Saccharomyces cerevisiae, Lactobacillus arabinosus,* and *Streptococcus hemolyticus,* Axelrod & Hofmann (133) found that a number of oxybiotin analogues had varying degrees of antagonistic activity against biotin and oxybiotin. Two of the more active antagonists were found to be inactive in organisms not requiring an exogenous source of biotin (*E. coli* and *S. aureus*). DL-Homooxybiotin was without effect in an infection of mice with a strain of *S. hemolyticus.* The absence of an effect was explained by the assumption that in the animal conditions prevail which keep the organism supplied with compounds (aspartic acid and fatty acids) which relieve the growth inhibitory effects of biotin antagonists.

Seits (134) demonstrated that 3-phosphoglyceric acid was not fermented by a thoroughly dialyzed macerated yeast juice. Adenylic acid, magnesium ions, and either potassium or ammonium ions were required. In the absence of either of the two latter ions, the reaction stopped at the phosphopyruvic acid stage. The negative results obtained by Muntz (13) with a similar preparation were said to be due to insufficient dialysis of the macerated juice.

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# NEWER ANTIBIOTICS[1]

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The newer antibiotics, for the purpose of this review, will include those antibiotics which have been introduced and investigated since the introduction of penicillin and streptomycin. However, any significant work which has appeared concerning penicillin and streptomycin since the last review of the antibiotics by Cutting (1) will be considered.

## PENICILLIN

Several hundred articles have appeared concerning penicillin alone. On the other hand, it could hardly be said that many of these articles have contributed anything new concerning the clinical aspects of this substance. To illustrate, the author has examined 75 articles which appeared during the last year on the general subject of penicillin in the treatment of syphilis. From this array of material, one could conclude that penicillin has established itself as an effective agent in the treatment of syphilis, a fact which was fairly well known five years ago.

Some experimental studies on penicillin worthy of comment have appeared since the subject was last reviewed. Investigators have concerned themselves with various studies of penicillin which might shed some light on the mode of action. On the other hand, it cannot be said that any precise knowledge of the action of this substance is presently available. Mills (2) reported some studies to suggest that the development of penicillin resistance of *Neisseria* could be explained on the expansion of a single enzyme rather than replacement of a partially blocked enzyme by another. That penicillin blocks the assimilation of glutamic acid by *Staphylococcus aureus* was suggested by the studies of Gale & Rodwell (3). This is not a new concept but an extension of studies previously reported by these same investigators. According to their studies, the effective concentration of penicillin is of the same order, regardless of whether or not the culture is sensitive or resistant to penicillin. These same investigators suggested that penicillin interferes with the mechanism whereby certain amino acids are taken into the cell. Plough & Grimm (4) reported studies that further support this contention. Pandalai & George (5) also summarized evidence suggesting that the action of penicillin is essentially an intracellular one.

It has been shown that the bacteriostatic effect of penicillin preparations persists for a number of hours after removal of the drug. On the other hand, the report of Eagle & Musselman (6) showed that there was no demonstrable correlation between the sensitivity of various organisms to penicillin and the rate at which the toxic effects were manifested.

[1] This review covers the period from January, 1949 through February, 1950.

The general subject of penicillin resistance will be reviewed elsewhere in the present volume. From a practical standpoint, however, it should be pointed out that the development of resistance on the part of bacteria is still of little clinical importance except in respect to *Micrococcus pyogenes*. Within a number of institutions, the incidence of penicillin resistance on the part of staphylococci appears to increase each year. In an examination of the general subject, Nichols & Needham (7) reported that the incidence of resistance was 68 per cent. This is slightly higher than the incidence reported by Barber & Rozwadowska-Dowzenko (8), as well as Rountree & Thomson (9). The latter investigators reported an incidence of 53 per cent of penicillin-resistant strains. The important fact in connection with these studies, however, lies in the observation that these organisms are penicillinase-producers, which is another way of saying that they have not become resistant but have always been resistant, provided, of course, one is prepared to accept the theory that the production of penicillinase is a property of organisms which have always been penicillin-resistant. Such a theory would support the notion that it is simply a matter of change in the bacterial population, rather than the development of resistance. The matter could be considered from the point of view of the widespread general use of the antibiotic with the elimination of the penicillin-sensitive strains, thereby allowing the penicillin-resistant (penicillinase-producing) variants to emerge.

Barber (10) reported some interesting studies on penicillinase-producing strains of *M. pyogenes* isolated from human infections. The study was designed to determine the permanence of the resistance to penicillin. Of 32 such strains tested for a number of months after isolation, 2 yielded penicillin-sensitive colonies, while 15 others yielded a few organisms which were sensitive. This phenomenon appeared to vary somewhat according to the conditions under which the cultures were kept. This tendency for some strains to yield penicillin-sensitive variant colonies was not accelerated by treatment with roentgen rays or by growing the strains with other organisms.

To refute the prevailing theory concerning spontaneous mutation, Eriksen (11) has reported some experiments which show that penicillin-resistant bacteria arise only after contact with penicillin. That growing penicillin-sensitive and penicillin-resistant organisms together may cause the penicillin-resistant organisms to become penicillin sensitive was suggested by George & Pandalai (12). Furthermore, they indicated that acquired sensitivity lasts for a fairly long time. They further suggested that the factor responsible for sensitivity is a very labile constituent of the bacterial cell which appears to be ribonucleic acid. The whole general subject of the mechanism of penicillin resistance, as well as the mechanism of action, needs further study.

Davis (13) has described a method for the isolation of biochemically deficient mutants of bacteria utilizing a procedure based on the fact that penicillin sterilizes only growing bacteria. By use of this technique, a large variety of interesting mutants of *Escherichia coli* was obtained.

Nothing very striking has been learned from recent publications concern-

ing absorption, diffusion, and excretion of penicillin. Of some interest is the report of Anderson & Brodersen (14) which indicated that bilaterally nephrectomized dogs were found to eliminate penicillin at a considerable rate from the blood stream. The major part of the penicillin was found to be excreted in the bile. The academic argument continues to rage concerning the advisability or inadvisability of intrathecal penicillin therapy. Such arguments stem from studies made to show the presence or absence of penicillin in the cerebrospinal fluid of normal subjects, as well as subjects with inflamed meninges. Boger & Wilson (15) restudied the general subject. They pointed out that, following single intravenous injections of large amounts of penicillin, therapeutically effective levels of penicillin are present in the cerebrospinal fluid of all patients studied. They concluded, therefore, that intrathecal injection of penicillin is unnecessary in the majority of patients. For reasons which will be mentioned later, the author does not agree. An interesting study on the penetration of penicillin into inflamed and normal membranes was that by Som and his colleagues (16). Their studies suggest that the addition of hyaluronidase to penicillin resulted in greater diffusion and penetration of penicillin, which was attributed to the spreading action of the hyaluronidase.

The use of penicillin preparations designed for prolongation of action has been the subject of considerable investigation. Procaine penicillin in sesame oil or procaine penicillin in an aqueous preparation is generally conceded to be the depot penicillin of choice. The author is in complete agreement with the report of Haunz & Grinnell (17) suggesting total abandonment of preparations of calcium penicillin in beeswax and peanut oil (Romansky formula). That there is no difference between the clinical response to be expected from procaine penicillin and the response from regular penicillin was clearly evident from the report of Griffiths and co-workers (18). Fischbach and his colleagues (19) reported studies which throw some light on the subject of procaine penicillin and its possible antagonism toward the sulfonamides. Their study clearly indicates that procaine penicillin may be safely administered simultaneously with sulfadiazine, sulfathiazole, and sulfapyridine. That the bacteriostatic activity of sulfadiazine in the serum of children receiving this sulfanomide was not affected by the administration of procaine penicillin was further evident from the study of Goldberg & Kagan (20).

The subject of discontinuous therapy with penicillin needs comment. While one or two daily doses of penicillin in aqueous solution will be found satisfactory in the treatment of uncomplicated pneumococcal pneumonia, this method of administration is inadequate for the treatment of severe generalized infections. This general comment finds support in the report by Tompsett and co-workers (21).

The somewhat controversial observations on the effect of penicillin on the coagulation of the blood received consideration in a report by Mosonyi *et al.* (22). They concluded that the effect of penicillin depends on the phase in which the substance is added to the system. They were of the opinion

that this observation brings the seemingly controversial reports by various authors into harmony. While the phenomenon is of some interest, it is the author's opinion that the effect is of no practical significance.

Penicillin prophylaxis of rheumatic fever and subacute bacterial endocarditis has received considerable attention during the past year. It is too soon to evaluate the effectiveness of the substance in the prevention of rheumatic fever. However, two significant reports have appeared on the possible indication for and value of the prophylactic use of penicillin or other antibiotics in individuals who have systolic murmurs or evidence of heart disease and who are to undergo surgical or instrumental diagnostic procedures. These reports were made by Lichtman & Master (23) and by Merritt (24) on the penicillin prophylaxis of bacterial endocarditis.

The intrathecal use of penicillin in the treatment of severe meningitis has been controversial. Adequate systemic therapy should routinely be combined with intrathecal therapy if good results are to be obtained in the treatment of meningitis, particularly pneumococcal meningitis. The reports of Appelbaum and co-workers (25), as well as that of Meneely (26), support this contention.

It is unfortunate that some investigators are recommending penicillin and other antibiotics, such as streptomycin, in the treatment of nocardiosis. In practically every case in which chemotherapy has been used successfully, the patient received adequate sulfonamide therapy. The report from the Mayo Clinic (27) is at variance with that by Hager and his colleagues (28) on the subject of therapy of infections due to *Nocardia asteroides*.

## Streptomycin

During the past year, dihydrostreptomycin has largely replaced streptomycin for clinical use. Unfortunately, the great problem of development of resistance remains the same with this hydrogenated form of the antibiotic. Reduced toxicity, therefore, is its only advantage. A considerable amount of work has been done on the production of streptomycin by different strains and mutants of *Streptomyces griseus*. Waksman & Harris (29), for example, have reported that the original culture of *S. griseus*, which had lost the capacity to produce streptomycin after 30 years in artificial media, would, when irradiated, yield a mutant that was a potent producer, in fact, as potent as the streptomycin-yielding strains of *S. griseus* isolated in 1943. Similar improvement in streptomycin-producing strains following use of ultraviolet as well as roentgen ray energy was reported by Savage (30). Similarly, Dulaney and his colleagues (31) pointed out that the normal variation found in *S. griseus* can be increased with ultraviolet light. This strain selection and induced mutation can be used to obtain strains with increased capacity for the production of streptomycin. A method was reported by Hosoya *et al.* (32) which would differentiate between streptomycin-producing and streptothricin-producing strains of *Streptomyces*. It is based on the fact that the growth of *Bacillus agri* was strongly inhibited by streptothricin and

was not inhibited by streptomycin. This microbe is, therefore, used to separate the different strains.

Some studies have appeared which concern themselves with the possible mode of action of streptomycin. It is interesting that for the most part streptomycin and dihydrostreptomycin are equally effective against most microbes. From the reports of Rake and his colleagues (33) it would appear, however, that various strains of *Salmonella* reveal a unique behavior. They found, for example, that in this genus, all strains tested were less sensitive to dihydrostreptomycin than to streptomycin. Conversely, it would appear that dihydrostreptomycin is somewhat more active than streptomycin in the treatment of eggs infected with *Rickettsia microti*. These observations are of interest, but it seems that, for the most part, the two substances behave in a like manner. That members of *Hemophilus influenzae*, type B, population are not uniformly sensitive to streptomycin was evident from reports by Alexander & Leidy (34). These same studies tended to show that in high concentration, streptomycin exerted a rapid lethal action. It appeared that the speed of the action was a corollary of the concentration of the antibiotic.

Gezon & Fasan (35) made certain observations on antigenic and enzyme system changes in beta hemolytic streptococci which were resistant to streptomycin. Their data suggest that, after acquiring streptomycin resistance, these organisms may demonstrate a reduction in enzymatic capacity or lose some antigenicity. While this same observation held true for penicillin resistance, a comparison revealed that resistance to aureomycin and bacitracin was associated with little change in activity as compared with the parent strain. Zeller and his colleagues (36) have made some observations of the influence of streptomycin and dihydrostreptomycin on some enzymes of *Mycobacterium smegmatis*. These studies clearly indicate that diamine oxidase of streptomycin-sensitive strains of *Mycobacterium* is inhibited by small concentrations of streptomycin and dihydrostreptomycin. Much larger concentrations were required to produce inhibition for streptomycin-resistant strains. In order to determine whether the influence of streptomycin and dihydrostreptomycin was specific for diamine oxidase, several other systems of enzymes were checked. None of them were affected.

Using streptomycin-sensitive strains of *E. coli*, Oginsky and his colleagues (37) found that streptomycin specifically inhibited an oxidative reaction in these organisms. The reaction was apparently the oxaloacetate-pyruvate condensation, which, when inhibited, prevents certain substances from entering the respiratory system. In another report, these same investigators (38) found that streptomycin-resistant and streptomycin-dependent variants of the same organism no longer possessed the ability to affect this condensation. In other words, the ability of the organism to grow in the presence of streptomycin apparently depends upon the development of unknown reactions which permit the cell to dispense with the condensation. Umbreit & Tonhazy (39) further reported that streptomycin will inhibit the oxaloacetate-pyruvate condensation in animal tissue, as well as in the bacterial

cell. The studies of Henry and co-workers (40) using *Shigella sonnei* showed that coincident with inhibition of multiplication by streptomycin, there was inhibition of the utilization of carbohydrate substrate and also inhibition of oxygen consumption per cell. Whether or not this interference is the primary cause of bacteriostasis or some indirect effect still, according to these authors, remains to be proved.

In spite of a huge bibliography on the general subject of streptomycin, very little new or significant information concerning new uses for streptomycin has appeared during the period which this review covers. Reports by Ross and his colleagues (41) and by Reid and his colleagues (42) fairly well indicate that, in spite of demonstrated susceptibility of *Salmonella* to streptomycin, the antibiotic has proved of little value in the treatment of *Salmonella* enteritis. Reports by Jacoby and co-workers (43), Stewart & Laur (44), and Sondag and his colleagues (45) would indicate that streptomycin has established itself as an effective agent in the treatment of granuloma inguinale. The latter report is based on 597 patients. Of those patients followed up, 92 per cent were free of open lesions at the time of last observation.

The value of streptomycin in the treatment of whooping cough seems controversial. Jongco (46) and his associates reported improvement in 90 per cent of patients who received the antibiotic, whereas Schwabacher and co-workers (47), Gordon & Almaden (48), and Wannamaker and his colleagues (49) all reported fairly convincing evidence that no significant improvement occurred in patients with whooping cough who received the antibiotic. That streptomycin was superior to other forms of therapy in the treatment of human plague was suggested by the report of Karamchandani (50).

## Aureomycin

It is clearly evident that aureomycin is the most important antibiotic developed since the introduction of penicillin. Considerable information concerning its activity and its usefulness has accumulated since the antibiotics were last reviewed in this publication. Broschard and his associates (51) described the properties of this antibiotic isolated from the substrate of *Streptomyces aureofaciens*. It was evident from their report that aureomycin is a weakly basic compound containing both nitrogen and nonionic chlorine. The substance forms a hydrochloride, which is the preparation most commonly used both experimentally and clinically. The substance decomposes at temperatures greater than 210°C.; $[a]D^{23°}, -240.0$ (water). Its solubility in water is 14 mg. per ml. at 25°C. The pH of the aqueous solution is 2.8 to 2.9. Analysis shows the composition on a percentage basis, to be as follows: carbon 51.84, hydrogen 5.24, nitrogen 5.46, total chloride 13.27, ionic chloride 6.69, and oxygen 24.19 (by difference).

*In vitro and in vivo activity.*—Demerec (52) reported some interesting studies suggesting that the pattern of aureomycin resistance is different from that of streptomycin resistance. He further pointed out that aureomycin resistance may be similar to penicillin resistance. These observations are in

line with the clinical experience to date with aureomycin, namely, that the development of resistance has not been a major problem.

That aureomycin and penicillin were more active against gram positive bacteria than are streptomycin or chloramphenicol was quite evident from the report of Pelcak and his co-workers (53). Against gram negative bacteria, aureomycin was the most active of the antibiotics tested. In other words, of the four antibiotics mentioned, aureomycin appears to be the most active against both classes of microbes. That aureomycin was not inhibited in the presence of blood or serum in the media was demonstrated by Beigelman (54). A comparison of the bacteriostatic effect of aureomycin with that of streptomycin against *Brucella* was reported by Lacy & Lankford (55). Under conditions of their experiment, no aureomycin-resistant mutants were selected from several strains of *Brucella*, in contrast to the fact that first-stage mutants with high tolerance to streptomycin were yielded by the species examined. Fuller & Faust (56) reported that strains of *Endamoeba histolytica* grow in cultures containing the optimal amount of aureomycin as well as, if not better than, these organisms will grow in penicillin-streptomycin cultures. Steenken & Wolinsky (57) examined the *in vitro* and *in vivo* activity of aureomycin against *Mycobacterium tuberculosis*. Under the conditions of their experiments, it is quite obvious that the antibiotic has no deterrent effect upon the course of tuberculous disease in guinea pigs. These same findings were confirmed by Perry (58). Both studies just referred to suggest that the guinea pigs do not tolerate aureomycin therapy well.

That aureomycin possessed potent antirickettsial properties was quite evident from the studies of Anigstein and his colleagues (59). The results of these studies are in agreement with the previously reported observations of Wong & Cox (60). Heilman (61) has reported a careful study of the action of aureomycin against *Brucella* infections in mice. It was evident from his studies that aureomycin had a very definite suppressive effect but that *Brucella* organisms were not completely eliminated from the infected tissues after the use of this antibiotic alone. After comparing aureomycin alone and in combination with a number of other antibiotics, he concluded that the most suppressive effect on *Brucella* was exerted by a combination of aureomycin and dihydrostreptomycin. That these original observations by Heilman were correct would seem to be indicated by similar studies subsequently reported by Magoffin and co-workers (62). The latter studies were carried out on *Brucella* infections in the developing chick embryo.

Ransmeier (63) and also Larson (64) reported studies which indicate that aureomycin is highly effective, both *in vitro* and *in vivo*, against *Bacterium tularense*. While these studies indicate that aureomycin should be investigated in the treatment of tularemia, it should be remembered that no therapeutic failures have occurred to date when streptomycin has been used. The advantage of aureomycin, however, would be that it can be effectively administered by the oral route. Bradford & Day (65) found that aureomycin in rather large doses protected mice against experimental infections owing to *Hemophilus pertussis*. However, this action was only bacteriostatic. Deaths

frequently occurred in the animals after cessation of therapy.

According to Wells & Finland (66), aureomycin appeared to be effective in the control of psittacosis infections in chick embryos. Eaton reported that aureomycin inhibited the growth of atypical pneumonia virus in chick embryos and that pulmonary consolidation was almost completely inhibited in cotton rats infected with the virus of primary atypical pneumonia (66a). There appeared to be a direct relation between the dose of the antibiotic and the prolongation of life. There is some evidence in this report to suggest that the prolongation of life following aureomycin therapy was a little longer than the effect noted with chloramphenicol. That aureomycin had a slight suppressive effect against toxoplasmosis in mice was suggested by the report of Summers (67). It was found in his experiment that *p*-aminobenzoic acid did not counteract the protective effect of aureomycin which can be interpreted as simply indicating that the mode of action of the antibiotic is different from that of the sulfonamides. Experimental melioidosis in guinea pigs was not influenced by treatment with aureomycin, according to Cruickshank (68). It was evident from the report of Baldridge and his colleagues (69) that aureomycin had no *in vitro* activity against the virus of herpes simplex. As compared with its activity against the larger viruses, no similar *in vivo* effect against herpes simplex could be demonstrated.

*Pharmacologic aspects.*—A suitable method for the determination of the aureomycin content of body fluids was first described by Dornbush & Pelcak (69a). Another satisfactory method for the determination of the aureomycin content of various body fluids has been described by Heilman & Herrell (70). Using this method, studies were made on the absorption, diffusion, and excretion of aureomycin following its oral and intravenous administration. Aureomycin is readily absorbed. In contrast to previously available antibiotics, the amount in the general circulation is maintained at a rather constant level for a number of hours. Repeated administration does not result in a piling-up effect. From these studies, it appears that aureomycin diffuses readily into the cerebrospinal fluid. It diffuses through the placenta and is available in the fetal circulation. Aureomycin also diffuses into the pleural fluid. It is concentrated in the normal hepatic system and is excreted in fairly high concentrations in the bile. After therapeutically effective doses, it was demonstrated in the tissues of the liver, kidney, spleen, and lung. Large amounts are constantly excreted in the urine of patients receiving the substance. According to Lepper and his colleagues (71), aureomycin could not be demonstrated in the milk, even in the presence of high concentrations in the blood. These investigators also reported studies to indicate that the material diffused into the cerebrospinal fluid. Some of the observations reported by Heilman & Herrell were further confirmed by Dowling and his colleagues (72). DeRoetth (73) reported some studies on the penetration of aureomycin into the eye. When compared with other antibiotics, aureomycin appeared to penetrate less readily into the eye, but once it had crossed the blood-aqueous barrier, it seemed to remain longer in the ocular fluids and tissues.

In connection with the absorption of aureomycin, the subject of aureomycin and preparations of aluminum hydroxide has received some attention. According to DiGangi & Rogers (74), aluminum hydroxide is capable of absorbing almost 100 per cent of the antibiotic as indicated by both bacteriological and colorimetric assays. Waisbren & Hueckel (75) reported a very marked dropping of the serum levels of aureomycin in patients who took preparations of aluminum hydroxide along with aureomycin. It is true that preparations of aluminum hydroxide do interfere with the absorption of aureomycin, but therapeutically effective concentrations can be attained when one uses 1 teaspoonful of aluminum hydroxide gel with each oral dose of 750 mg. of aureomycin. In any event, it is not necessary to use preparations of aluminum hydroxide in more than 10 to 20 per cent of patients.

In addition to the method described by Heilman and the author for the estimation of aureomycin in various body fluids, a number of procedures have been recently described. It is impossible to assess the value of one method versus another. It would seem that the procedure should be adopted which is most suitable for the particular laboratory in which these assays are being carried out. Methods have been described by Brainerd and co-workers (76), Schneierson & Toharsky (77), Levine and co-workers (78), Randall *et al.* (79), Whitlock and co-workers (80), and Saltzman (81).

*Toxicity and reactions.*—Like penicillin, aureomycin is remarkably free of serious toxic effects, In spite of widespread clinical experience of nearly two years' duration, only two reports have appeared at the time of this writing describing toxic cutaneous effects of aureomycin in two separate cases. One case was reported by Riese (82) and another case by Rosanova & Warszewski (83).

Macht & Farkas (84) suggested that aureomycin may interfere with the coagulation of the blood and thereby favor thrombo-embolic accidents. While these observations are of interest, the hazard of the use of aureomycin on this basis is of little or no practical significance. Randall and his colleagues (85), reporting on the intravenous administration of aureomycin, found that when one exceeds the recommended dose of 0.5 gm. of aureomycin by the intravenous route, certain mild toxic effects may be experienced. When as much as 1 gm. of aureomycin is administered intravenously at a single injection, on occasion nausea and vomiting may occur and mild febrile reactions may be induced.

*Clinical investigations.*—The results of an enormous amount of clinical investigation on aureomycin have appeared in a short time. At the present time, aureomycin is considered by many investigators the treatment of choice in most rickettsial infections, lymphogranuloma venereum, granuloma inguinale, herpes zoster and primary atypical (virus?) pneumonia. (It is by no means effective in all infections of virus origin.) It is considered effective in many infections owing to gram negative microbes such as *Klebsiella*, *Hemophilus influenzae*, and *Pasteurella tularensis*. It is considered to be of some value in the treatment of brucellosis. Certain coccal infections which are resistant to penicillin are said to respond. This is especially true

of penicillin-resistant staphylococcal infections, as well as infections owing to *Streptococcus faecalis*. That it possesses definite antispirochetal activity is suggested by reports which are also available.

The average daily dose of aureomycin by the oral route in the treatment of moderately severe infections is 3 gm. in adults. A satisfactory preparation of aureomycin for intravenous therapy has been described somewhat in detail by Sanders and co-workers (86) and also by Randall and his colleagues (85).

It is difficult from the examination of published material to assess the value of aureomycin in the treatment of infections of virus origin. The report of Wright and co-workers (87) clearly indicated that aureomycin was of value in the treatment of lymphogranuloma venereum. This report was amply confirmed by Robinson and co-workers (88) and by Prigot *et al.* (89). It should be remembered, however, that the organisms of the lymphogranuloma-psittacosis group are borderline and may not remain in the classification of true viruses. On the other hand, rather convincing evidence concerning the clinical effectiveness of aureomycin in the treatment of herpes zoster was presented by Case (90), by Binder & Stubbs (91), and by Finland and his colleagues (92). Concerning the possible value of aureomycin in infections owing to herpes simplex, it is difficult to make compatible the experimental studies discussed above and the clinical reports of Baer & Miller (93), Bereston & Carliner (94), and Arnold and co-workers (95).

There appears to be general agreement among a number of investigators (96 to 104) that aureomycin exerts a very definite and beneficial effect on the course of so-called viral or primary atypical pneumonia. One should bear in mind, however, that the status of the virus of so-called atypical pneumonia has, until recently, been somewhat uncertain. The published data contain little to support the value of aureomycin in certain important virus infections, such as the common cold, influenza, poliomyelitis, encephalitis, variola, and rubella. Evidence to date concerning its value in the treatment of plantar wart is by no means convincing.

In aureomycin, it would appear that an effective agent is at hand for the treatment of the rickettsial diseases including Q fever, typhus, and Rocky Mountain spotted fever. That Q fever was effectively treated was evident from the reports of Lennette and co-workers (105). It was evident from the report of Ross and his associates (106) that aureomycin was more effective than *p*-aminobenzoic acid in the treatment of Rocky Mountain spotted fever. Similar excellent results have been reported by Harrell and co-workers (107), by Gear & Harington (108), and by Baker (109). The report of Knight and his colleagues (110) leaves little doubt concerning the effectiveness of aureomycin in the treatment of typhus.

A number of reports have appeared concerning the value of aureomycin in the treatment of infections of the urinary tract and also on the use of aureomycin in the treatment of several important venereal diseases. Collins & Finland (111) were impressed with the value of aureomycin in the treatment of certain urinary infections. Carroll and his colleagues (112)

concluded that aureomycin was effective in the treatment of urinary infections owing to *Escherichia coli* and *Aerobacter aerogenes*, paracolon bacillus infections and infections owing to *Streptococcus faecalis*. Obviously, aureomycin, on the other hand, is no substitute for the elimination of foci and other abnormal conditions, including obstruction, which may be present in infections of the urinary tract. Most investigators are in agreement that aureomycin is of little value in the treatment of infections owing to *Pseudomonas aeruginosa* and *Proteus*. In favor of aureomycin over streptomycin is the fact that bacterial resistance does not readily occur. Rutenburg & Schweinburg (113) reported no evidence of the development of bacterial resistance in a series of infections of the urinary tract.

The reports of Hill and co-workers (114), Robinson and colleagues (115), Prigot and associates (116), and Greenblatt and co-workers (117) contain information on the use of aureomycin in the treatment of 83 patients suffering from granuloma inguinale. While streptomycin in the past has been reported to be of some value in the treatment of this infection, the results of the studies just mentioned leave one with little doubt that aureomycin is far more desirable for this purpose. Chen and his colleagues (118) reported good therapeutic results with aureomycin in the treatment of gonorrhea. On the other hand, no evidence is at hand to suggest the advisability of substituting aureomycin for penicillin in the treatment of infections owing to *Neisseria gonorrhoeae*. In fact, the report of Robinson (119) would suggest that rate of cure with aureomycin may be less than that with penicillin. That aureomycin was of value in the treatment of chancroid was suggested by the report of Zheutlin & Robinson (120).

The experimental studies of Heilman (121) clearly suggested that aureomycin possessed considerable antispirochetal activity. Other studies from the Mayo Clinic (122, 123, 124) clearly demonstrated that aureomycin was of considerable value in the treatment of early, late cutaneous, and central nervous system syphilis. Admittedly, penicillin is still effective in the treatment of syphilis. On the other hand, aureomycin supplies a new antibiotic for the treatment of this infection and moreover, may be employed for patients whose condition is resistant to penicillin and patients who are sensitive to penicillin. There is some evidence to suggest that aureomycin may be superior to penicillin in the treatment of syphilis of the central nervous system. Similar observations concerning the effectiveness of aureomycin in the treatment of syphilis have been reported by Irgang & Alexander (125), by Wiggall and his co-workers (126), by Rodriquez & Plotke (127), and by Willcox (128).

The problem of penicillin resistance on the part of microbes which were originally found to be susceptible to its action has been grossly exaggerated and overemphasized. There is one exception to this general statement. The number of strains of staphylococci resistant to penicillin which are being encountered has steadily risen during the past few years. The general subject of resistance on the part of staphylococci has been discussed elsewhere in this review. Of the penicillin-resistant strains of staphylococci examined

by Nichols & Needham (7), all were found to be quite sensitive to the action of aureomycin. Clinical reports by these investigators as well as reports by Chandler and co-workers (129) clearly indicated that aureomycin is effective in the treatment of infections owing to these penicillin-resistant strains of *M. pyogenes*.

That aureomycin had a definite suppressive effect upon the course of culturally proved brucellosis was evident from the reports of Spink and his associates (130), as well as from the reports of Bryer and co-workers (131) and Knight and colleagues (132). Review of subsequently published data, including those of Spink & Yow (133), indicates that aureomycin, on the other hand, is by no means a specific or uniformly curative agent. There can be little doubt that the most effective method to date of treatment of culturally proved brucellosis is that described by Herrell & Barber (134) in which aureomycin is combined with dihydrostreptomycin.

McVay and co-workers (135) and MacDonald (136) have reported on the successful use of aureomycin in the treatment of amebiasis. These observations are interesting but difficult to bring into line with the *in vivo* studies mentioned earlier in this review in which aureomycin was successfully used to cultivate *Endamoeba histolytica* unless one postulates that cure of human amebiasis is contingent upon the eradication of the bacteria from the intestinal flora, some of which are essential for the support of the amebae, which can no longer survive in the relatively sterile intestinal tract. A very comprehensive study on the effect of aureomycin on the bacterial flora of the intestinal tract of man has been reported by Dearing & Heilman (137). It is clearly evident from their studies that aureomycin is the most effective agent available to date for removing bacteria from the intestinal tract. All bacteria culturable by the methods used by these investigators except *Proteus* and *Pseudomonas* were removed from the intestinal tract under the conditions of one of their experiments. Even species of *Bacteroides*, which are present in large numbers in normal feces, were not found in the feces of five patients who received aureomycin. In five untreated patients, numerous *Bacteroides* microbes were found in the fecal specimens under similar cultural circumstances. This is a real contribution to the preoperative preparation of patients. According to these investigators, the conditions which hindered or prevented removal of susceptible bacteria were perforated intestinal lesions, intestinal fistulas, and intestinal obstruction.

Bell and his colleagues (138) suggested that aureomycin exerts a beneficial effect upon the course of experimental and also human pertussis. On the other hand, Schoenbach (139) stated that aureomycin does not appear to be effective in whooping cough. To date, the matter is unsettled. The experimental studies referred to earlier concerning the possible value of aureomycin in the treatment of tularemia find confirmation in the clinical reports of Woodward and co-workers (140). Ransmeier and his colleagues (141), Carroll & Gorman (142), and Lindeke & Maiden (143), all of whom reported on the successful use of aureomycin in the treatment of human tularemia. Aureomycin, however, can hardly be expected to surpass the results already ob-

tainable by use of streptomycin in the treatment of this infection. The lack of toxicity and the ease of oral administration, however, probably favor the use of aureomycin.

Aureomycin has been investigated in the treatment of a number of infections of the skin. It has been used systemically and also in the form of an ointment. Various types of skin lesions have been reported to show favorable response. For example, Hollander & Hardy (144) were impressed with the value of the substance in the following types of conditions: infected ulcers, dermatitis, pyoderma, necrosis of the skin, pustular psoriasis, and exfoliative lesions. According to Robinson *et al.* (145), a temporary suppressive effect was observed in dermatitis herpetiformis. Some evidence to support the notion that aureomycin was of value in the treatment of pemphigus appeared in the reports by Philip (146), Natenshon (147), and Bettley (148). Final assessment of the value of aureomycin in the treatment of such a variety of infections must await more extensive studies. That aureomycin may prove to be of value in the treatment of certain ocular infections including dendritic keratitis is suggested in the reports of Appelman & Hale (149) and Braley & Sanders (150).

The possible value of aureomycin in the treatment of a number of miscellaneous infections must be considered. It was suggested by Gruskin (151) and Lyons (152) that aureomycin may be of value in the treatment of infectious mononucleosis. Unfortunately, a large, well-controlled study of the general subject has not yet appeared. The wide range of antibacterial activity of aureomycin, which includes its effectiveness against both gram negative and gram positive microbes as well as species of *Clostridium* commonly found in the intestinal tract, immediately suggests the value of this antibiotic in the prevention and treatment of acute suppurative peritonitis. The studies of Yeager and co-workers (153) on experimental peritonitis in dogs revealed the rather exciting result of a 90 per cent survival rate in dogs treated with aureomycin, as compared with a 20 per cent survival rate in the control animals. Their studies, as well as those of Wright and his colleagues (154), in spite of the fact that the clinical experience was not large, are impressive enough to establish aureomycin as the antibiotic of choice in the treatment of peritonitis. McVay and co-workers (155) have reported two cases of septicemia due to *Bacteroides* in which the disease was successfully treated by the oral administration of aureomycin.

In view of the experimental studies previously discussed, it is not surprising that Steinbach and co-workers (156) found aureomycin of little or no value in the treatment of pulmonary tuberculosis. The temporary clinical improvement in the pulmonary involvement of pancreatic fibrosis following aureomycin therapy, which was noted by Shwachman and his colleagues (157), is probably best explained on the basis of the control of secondary infection, rather than any effect upon the primary disease itself. A uniform disappearance of pleuropneumonia-like organisms from the genitourinary tract of individuals was reported following the oral administration of aureomycin by Brown and co-workers (158). This occurred in patients

with and without disease of the joints, and the disappearance of the L organism occurred after the administration of aureomycin, whereas gold therapy had not eliminated the organism. No conclusions can be drawn at this time concerning the possible value of aureomycin in the treatment of rheumatic diseases. It might be pointed out, however, that using aureomycin, Kuzell and co-workers (159) were able to cure experimental polyarthritis in rats due to the L4 strain of pleuropneumonia-like organism. On the other hand, it is not surprising that aureomycin produced no beneficial effects in several patients with chronic rheumatoid arthritis.

Published studies to date dealing with aureomycin leave one with the inescapable impression that this antibiotic is probably the most versatile and most important antibiotic developed since the introduction of penicillin.

## Chloramphenicol (Chloromycetin)

Ehrlich and his associates (160) have described *Streptomyces venezuelae* which produces chloramphenicol. It is somewhat similar to but quite distinct from *Streptomyces lavendulae*, the organism which produces streptothricin. To the chemist, chloramphenicol, as compared with other antibiotics, has been a genuine delight. It is the first antibiotic of importance which can be synthesized with relative ease. Rebstock and co-workers (161) determined the chemical structure of chloramphenicol to be D-(−)-threo-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol. This same group of investigators (162) pointed out that one of the isomers of the synthesized product was found to correspond in all ways to the fermentation-produced substance. Details on the procedure of synthesis of chloramphenicol have been described by Long & Troutman (163, 164). That the synthesized and the fermentation-produced substance behaved in the same manner in the treatment of experimental infections was further evident from the report by Smadel and his associates (165).

*In vitro and in vivo.*—One of the most extensive reports on the antimicrobial activity of chloramphenicol is that by McLean and his colleagues (166). They examined the susceptibility of 64 genera of microorganisms, including 290 species and strains. All of the *Rickettsia* tested were found to be susceptible to chloramphenicol. It was suggested, further, that the lymphogranuloma venereum-psittacosis group of viruses may prove susceptible. The results of tests on the effect of this substance on the virus of poliomyelitis were reported as negative. In the report just mentioned, it was concluded that under the conditions of the test, chloramphenicol might find usefulness in the treatment of a wide variety of bacterial infections, including those caused by the genera *Aerobacter*, *Brucella*, *Diplococcus*, *Escherichia*, *Hemophilus*, *Klebsiella*, *Micrococcus*, *Neisseria*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Streptococcus*, and *Vibrio*. The report by McLean and co-workers further suggested that chloramphenicol possessed little or no activity against the fungi. Of 40 species studied, only 2 were inhibited in the concentrations used in these tests. With the exception of *Borrelia recurrentis*, the results of studies on the spirochetes tested were considered

not very promising. That chloramphenicol was effective *in vivo* in the control of experimental infections owing to *Vibrio comma* was suggested by the report of Gauld and his associates (167).

A detailed study of the rickettsiostatic effect of chloramphenicol in eggs infected with *Rickettsia tsutsugamushi*, *Rickettsia mooseri*, *Rickettsia rickettsi*, *Rickettsia akari*, *Rickettsia prowazeki*, and *Rickettsia burneti* has been reported by Smadel and co-workers (168). Beneficial effects were noted in experimental infections caused by the first four named *Rickettsia* in mice and also in guinea pigs. It is very significant that treatment was of value in all of these experiments, even when therapy was delayed for appreciable periods of time after infection.

In spite of the fact that chloramphenicol had been found to inhibit cocci *in vitro*, Bliss & Todd (169) reported the agent to be of no value in the treatment of peneumococcal and streptococcal infections in mice. On the basis of such studies, one would be reluctant to attempt clinical trials against these infections when other suitable agents are available. That chloramphenicol was probably the antibiotic of choice in the treatment of infections owing to *Salmonella*, including *S. typhosa*, was suggested by Alexander and her colleagues (170). Likewise, they presented data to suggest that the antibiotic is highly effective against infections owing to *Hemophilus influenzae* and *H. pertussis*. That little could be expected from chloramphenicol against *Pseudomonas aeruginosa* also was suggested by these studies.

Yow & Spink (171) found chloramphenicol a little less active on a weight-for-weight basis than aureomycin against various strains of *Brucella*. The concentrations of aureomycin required to inhibit varied between 0.6 and 1.5 μg. per ml., as compared with concentrations of chloramphenicol of 1.56 to 6.25 μg. per ml. In a comparison of aureomycin, chloramphenicol, penicillin, and streptomycin *in vitro*, it appeared from the studies of Pelcak and co-workers (172) that aureomycin and penicillin were more active against gram positive microbes than was either chloramphenicol or streptomycin. Against gram negative bacteria, aureomycin was generally the most active of the antibiotics tested. The published data available at present clearly indicate, therefore, that chloramphenicol is an active and rather polyvalent antibiotic, but the evidence does not suggest it as being superior to penicillin or aureomycin. One notable exception to this statement has to do with infections owing to *S. typhosa*, which will be discussed later.

*Pharmacologic aspects.*—Considerable information concerning the tissue distribution and excretion of chloramphenicol has been supplied by Glazko and co-workers (173). Chloramphenicol appears to be rather rapidly absorbed. Approximately 90 per cent of the material is excreted by way of the kidneys. It is interesting that a large portion of the excreted drug, according to these investigators, is in the form of an inactive nitro compound. Furthermore, less than 10 per cent of the material administered is excreted unchanged. It is further interesting that that which is excreted into the intestine by way of the bile is also excreted principally as inactive nitro compounds. That inactivation of chloramphenicol occurs in the liver is suggested by these

same studies. Inactivation, for example, occurred *in vitro* with preparations of minced liver. Finally, it was evident that the distribution of chloramphenicol in the tissues is not uniform. While high concentrations were found in the liver and kidneys, low concentrations were found in the brain and spinal fluid. This behavior of chloramphenicol is in striking contrast to the previously mentioned studies on aureomycin. In the treatment of certain infections, for example, infections of the biliary tract, these findings are of considerable practical importance.

Further studies on the pharmacologic behavior of chloramphenicol were reported by Gruhzit *et al.* (174). These studies suggested that chloramphenicol disappears rather rapidly from the blood stream. Furthermore, these studies showed that chloramphenicol is a relatively nontoxic substance for experimental animals. For example, 50 mg. per kg. of body weight was found to be well tolerated. No cumulative toxic effects were noted. One is inclined to agree with Crooks (175), who made the observation that with the knowledge of the fact that in chloramphenicol one is dealing with a nitrophenol compound, it did not seem reasonable that chloramphenicol should be nontoxic. As the matter stands, however, careful studies indicate that it is relatively nontoxic.

*Toxicity and reactions.*—From the standpoint of clinical investigation, the lack of toxicity of chloramphenicol finds considerable support in the fact that only one instance of cutaneous toxic reaction has appeared since its introduction. This reaction took the form of giant urticaria following administration of the substance to a patient suffering from typhoid fever. This case was reported by Sacks (176).

*Clinical investigations.*—In spite of the apparent polyvalence of chloramphenicol, the clinical investigations reported to date deal mainly with the effectiveness of the antibiotic in the treatment of rickettsial diseases and typhoid fever. According to some studies, it appears to have a suppressive effect on the course of brucellosis. The early investigations of Woodward and his colleagues (177) suggested that chloramphenicol exerted a specific therapeutic effect on the course of typhoid fever. In this regard, chloramphenicol is unique among antibiotics known at the present time. Although the substance resembles aureomycin in some respects, it is far superior in the treatment of typhoid fever. During the past year, 16 clinical reports (178 to 193) dealing with single and multiple cases leave one with the conviction that the course of typhoid fever is very favorably affected by this antibiotic. The general subject has been well summarized in a recent report by Woodward and his associates (194). Of considerable interest is the fact that, according to these investigators, none of the patients who received chloramphenicol in adequate doses became chronic typhoid carriers. This is, indeed, fortunate, since this, as well as other reports, clearly indicate that the experience with chloramphenicol in the treatment of the typoid carrier state has by no means been a happy one. Intensive therapy for two weeks failed to eradicate the carrier state, according to these investigators. Briggs (195) reported a relapse in typhoid fever after treatment with chloramphenicol.

However, it would appear that complete recovery followed the administration of a second course. The first course of treatment was one of five days. This relapse following a five-day course of therapy is not surprising in view of the definite conclusions of Smadel and his associates (196), who recommended that if relapses are to be avoided, chloramphenicol should be administered in fairly adequate amounts for at least more than eight days.

Clinical investigations continue to support the early reports on the effectiveness of chloramphenicol in the treatment of scrub typhus and also endemic typhus. Two very convincing reports concerning the chemoprophylaxis of scrub typhus have been made by Philip and his associates (197) and by Smadel and co-workers (198). The results of prolonged administration of chloramphenicol as a prophylactic agent as well as an agent for therapy of scrub typhus indicate that the drug is of low toxicity, and from these studies, it was further suggested that drug-fast strains of *Rickettsia* are not readily produced. The observations of Garvey and his colleagues concerning the emergence of resistant strains of gram-negative bacteria in patients under treatment with chloramphenicol may be of importance (198a).

The previously reported effectiveness of chloramphenicol in the treatment of Rocky Mountain spotted fever finds confirmation in the report by Carson and his associates (199). Woodward and co-workers (200) claimed a specific beneficial effect from chloramphenicol in the treatment of active brucellosis. Ralston & Payne (201) reported the relief of symptoms in 35 of 40 patients believed to have chronic brucellosis. In general, it is the author's opinion that these results are inferior to those obtained from the combined use of aureomycin and dihydrostreptomycin in the treatment of this infection.

Included among a group of miscellaneous infections in which chloramphenicol has been tried is infectious mononucleosis (202, 203, 204). Further studies seem indicated before any final conclusions can be made concerning chloramphenicol in this disease. Payne and his colleagues (205) claimed a beneficial effect from chloramphenicol in the treatment of pertussis. Encouraging response was reported in epidemic infantile gastroenteritis after the use of chloramphenicol by Rogers and co-workers (206). In this particular epidemic, sulfonamide compounds, penicillin, and streptomycin had resulted in no therapeutic effect.

It appears from what has gone before that chloramphenicol has clearly established itself in the treatment of typhoid fever and rickettsial diseases. If it is superior to other available antibiotics in other infectious diseases, it is not apparent from the available published data at present.

## Terramycin

In January, 1950, the group of investigators at the research laboratories of Charles Pfizer and Company, Incorporated (207), reported the isolation of a new antibiotic agent which may well be of considerable importance. This antibiotic, named terramycin, is elaborated by a strain of *Streptomyces rimosus*. From the published data, it appears to have an antibacterial spec-

trum not unlike that of aureomycin. It is an amphoteric substance and forms a crystalline hydrochloride and sodium salt. It appears to be stable over long periods in aqueous solutions at a pH of about 2 to 5. Chemical analysis reveals the following composition on a percentage basis: carbon 53.05, hydrogen 5.91, nitrogen 5.64, and oxygen (by difference) 35.4. The antibiotic shows a fairly low degree of toxicity in experimental animals. It also displays rather marked chemotherapeutic activity *in vivo*. Like aureomycin, it is effective by both the oral and parenteral routes of administration. In addition to its antibacterial activity, it appears to have antirickettsial activity, and in high concentration, it was suggested that there was some inhibition of infection in chick embryos with the PR8 strain of influenza A virus. Clinical reports on the use of this substance have not appeared at the time of this writing, although such studies are under way.

## Bacitracin

There can be little doubt that this substance is fairly active against a number of microbes. On the other hand, evidence continues to accumulate which suggests that the usefulness of this substance is rather restricted because of its nephrotoxicity. A comparative study of the renal damage caused by bacitracin produced by surface culture as well as deep-tank culture methods revealed, according to Smith and his colleagues (208), no significant statistical difference in nephrotoxicity with the various batches of bacitracin. The systemic administration of bacitracin to human subjects in amounts of 196,000 to 200,000 units daily over periods of 4 to 13 days was followed by moderate to severe diminution in the rate of glomerular filtration, and there was impairment of other renal function according to the report of Zintel and his colleagues (209). Likewise, Michie and associates (210) stated that in all but one of a group of patients receiving bacitracin in whom chronic studies were made, there was moderate to very severe reduction in all of the renal function studies employed. In view of the number of highly effective nontoxic antibiotics available, such studies as those just mentioned should cause one to ponder before using this substance systemically, except under most unusual circumstances. There can be little doubt that bacitracin can be used safely as a topical agent in the treatment of certain pyogenic infections. Good therapeutic results in pyogenic infections of the skin and other soft tissues have been reported by a number of investigators (211 to 215). That bacitracin may be safely and effectively employed in the form of an aerosol for the treatment of diseases of the pulmonary apparatus was suggested by Prigal & Furman (216). It would appear that no absorption of bacitracin into the general circulation occurs following this method of administration.

## The Polymyxins

An examination of the data reported by Bliss & Todd (217) suggests that most gram negative bacilli are considerably more sensitive *in vitro* to polymyxin than to aureomycin, chloramphenicol, or streptomycin. Unfortunately however, it would appear that the administration of polymyxin is associated

with certain undesirable toxic reactions. Stansly (218) expressed the opinion that certain toxic effects, especially the renal effects, were rather unconstant and more or less transitory. He suggested that the purity of the preparation may be of considerable importance. No convincing reports as to the clinical usefulness of polymyxin A have appeared. Ross and his associates (219), on the other hand, describe certain toxic manifestations associated with the use of polymyxin B (aerosporin), which, to say the least, are disturbing. Included among the undesirable reactions noted were leukocytosis, eosinophilia, evidence of renal damage, and considerable pain at the site of injection. That the prolonged use of polymyxin B produces rather serious toxic manifestations in the nervous system was reported by Jawetz & Coleman (220). Likewise, neurotoxic phenomena were observed by Pulaski and his associates (221) following the administration of both polymyxin B and polymyxin A. However, they did find the substance useful as a topical agent, and it was also thought to be a rather potent intestinal antiseptic. It is the opinion of the author that polymyxin seems to follow the pattern of most other antibiotic substances of bacterial origin, namely, that in spite of a high degree of antibacterial activity, their toxicity is a serious handicap to their systemic use.

## Miscellaneous Antibiotics

The most casual review of the general subject of antibiotics reveals a number of interesting substances. On the other hand, many of these substances are not of sufficient importance to justify detailed consideration. For the purpose of considering those which are active or semiactive at the moment, they will be grouped under the general subject of Miscellaneous Antibiotics.

An antibiotic substance to which the name "streptocin" has been given and which is said to be active against *Trichomonas vaginalis* and certain bacteria has been described by Waksman and his colleagues (222). The substance was isolated from the mycelium of *S. griseus*. Other than a description of the substance, up to the time of this writing no other information concerning its activity was available. A streptothricin-like antibiotic termed "$EI_8$" was described by Weiser and his associates (223). It was obtained from an unidentified *Streptomyces*. It is said to be bacteriostatic for a strain of *M. tuberculosis* in dilutions of 1:100,000.

Biocerin is an antibiotic which is apparently elaborated by *Bacillus cereus*. It has been described by Johnson *et al.* (224). Single injections of 20 mg. of the crude substance produced no fatalities in a group of mice. It has antibacterial activity against various gram negative and gram positive microbes. Alvein is an antibiotic derived from a strain of *Bacillus alvei*. According to Gilliver and his associates (225), it is a strongly basic polypeptide. In spite of its high activity against gram positive bacteria, it is hemolytic. Like many other similar antibiotics of bacterial origin, it probably has little future value.

Michener & Snell (226) described two antifungal substances, one termed the rhizoctonia factor and the other the *Aspergillus* factor, which were

obtained from cultures of *Bacillus subtilis* grown for the production of subtilin. These substances are, however, quite unlike subtilin. A fungistatic substance to which the name "mycosubtilin" has been given was also described by Walton & Woodruff (227). This, too, is derived from *B. subtilis.* An antibiotic substance with the ability to inhibit *Corynebacterium diphtheriae* in a dilution of 1:512,000 has been described by Miller & Rowley (228). It is obtained from a strain of *Bacillus mesentericus* isolated from the soil. Jameson (229) described an antibiotic elaborated by *Pfeifferella whitmori.* It was said to be active against *M. tuberculosis* and was also said to resemble penicillin. Encouraging results in small scale studies on experimental infections with diphtheria in the guinea pig were reported.

A substance which is said to be remarkable for its inhibitory effect on the growth of many pathogenic fungi has been described by Hobby and coworkers (230). The substance is termed "antibiotic XG." Depending upon the conditions of the experiment, it is said to be both fungistatic and fungicidal. The substance is produced during the growth of an unidentified organism which appears to be closely related to *B. subtilis.* It is a strongly hemolytic compound *in vitro.* This property would discourage its administration by parenteral routes. On the other hand, according to the report of the investigators who described the substance, preliminary clinical trials using the substance as a topical agent in the treatment of certain fungous infections show considerable promise.

The name "circulin" has been ascribed to two substances which, as was pointed out by Garson and co-workers (231), differ from each other in their chemical and biologic properties. Both antibiotic substances apparently are derived from members of the *Bacillis circulans* group. Henceforth, the name "circulin" is retained for the product from strain Q-19. The substance appears to be similar to the polymyxins. The term polypeptin is applied to the antibiotic called "circulin" by the V. D. Research Laboratory group. Circulin is hemolytic and extremely toxic, as was pointed out by McLeod (232). To date, efforts to reduce its toxicity and at the same time preserve its antibacterial activity have not met with success.

Neomycin is a new antibiotic substance of current interest. It was described by Waksman & Lechevalier (233). It is obtained from filtrates of *Streptomyces fradiae.* It is a water-soluble, thermostable, basic substance. The main interest in the substance is the fact that it is said to be active against streptomycin-resistant microbes, including the resistant strains of *M. tuberculosis.* A subsequent report by Waksman and his colleagues (234) revealed that the ratio between toxicity and therapeutic activity of neomycin in mice was in the order of 20:50. That neomycin was more effective than streptomycin against experimental infections caused by *Micrococcus pyogenes* and various *Salmonella* including *S. typhosa* was suggested by the group of investigators at Rutgers (235). Whether or not neomycin will prove effective in clinical trials is not evident from available data.

Although a number of antibiotic substances of plant origin have been described, this review has been restricted to antibiotics of microbial origin.

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# GENETICS OF MICROORGANISMS[1]

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Extension of the methods and concepts of genetics to the realm of microbiology has progressed with unprecedented rapidity during the past decade. At present, the materials and methods of microbiology are contributing increasingly to the attack on fundamental problems of heredity, and genetics has already helped to clarify many previously obscure phenomena of microbial reproduction, variation, and adaptation and has furnished effective tools for the pursuit of physiological and biochemical investigations.

An ever-increasing number of reviews has appeared since 1940, beginning with a symposium on the genetics of pathogenic organisms (81). Some of these have been general in nature (79, 85, 100, 138), while others have been concerned with special groups—viruses (6, 31, 47, 48, 87, 89, 129, 131, 198, 231), bacteria (27, 58, 141, 153), fungi (15, 80, 148, 149), and protozoa and algae (115, 157, 224 to 229), or with problems of broad biological interest to which microbial genetics has contributed, e.g., gene action and biosynthesis (14 to 17, 23, 33a, 236, 237, 258), mutation (9, 10, 50, 180), genetic effects of radiation (18, 33, 35, 135, 265), adaptive enzymes (174), resistance and infectivity (51, 89, 90, 152), bacterial transformations (21, 161), and immuno-genetics (32, 97, 114). Methods, both biological and physical, have been summarized comprehensively (163).

## Mechanism of Variation

Knowledge of the mechanism of variation in microorganisms, particularly in asexual forms such as bacteria, has increased rapidly. To evaluate inherited variation precisely, it is important to distinguish between nuclear and cytoplasmic change, between gene mutation and chromosome aberration, and between back-mutation at a given locus and suppressor mutation at a second locus masking the first mutant gene, and to determine whether similar phenotypes represent mutations at the same gene locus or are nonallelic. This is ordinarily possible only with sexual organisms, where classical genetic criteria are available for distinguishing the various possibilities, but new approaches and techniques are overcoming some of the limitations of asexual forms.

Whether an inherited variation is due to nuclear or to extrachromosomal change can be readily determined only in sexual organisms. There are relatively few instances of heritable characters which can be convincingly attributed to cytoplasmic factors (32a), and extrachromosomal inheritance in microorganisms, as in other forms, may well prove to be exceptional,

[1] This review covers primarily the period from January, 1949 to February, 1950.

perhaps because of the selective advantage conferred by a chromosomal mechanism which provides a basis for the maintenance and regular transmission of essential genetic units. The situation in *Paramecium* with regard to the cytoplasmic basis of kappa and of antigenic types has been summarized (228, 229). Nitrogen mustard can inactivate kappa in this organism (77) and can apparently cause other changes in cytoplasmic factors (78). Another clear case of extrachromosomal transmission has been reported in yeast (64, 65, 66, 221, 222, 240). A small-colony variant of *Saccharomyces*, produced by acriflavin treatment (and proved not to involve change of a chromosomal gene), is characterized by absence of cytochrome-oxidase and succinic-dehydrogenase activity, suggesting a certain degree of autonomy in reproduction of formed bodies in the cytoplasm which are known to carry these and other enzymes. Alteration or loss of a particular self-reproducing cytoplasmic entity is suggested also in the permanent disappearance of chlorophyll following streptomycin treatment of *Euglena* (159, 207). In *Neurospora*, an extensive search for cytoplasmic inheritance either of morphological or biochemical characters has so far failed to give any evidence of its existence. These experiments involved isolation of treated uninucleate microconidia which were tested for mutant characters before having been passed through a cross (239). Cytoplasmic inheritance of differences in pathogenicity has been demonstrated for another of the rust-fungi (118).

Suppressor mutations that affect the synthesis of methionine (83), the synthesis of pyrimidines (111), and the requirement for sulfonamide (62) are now known in *Neurospora*. Production of pigment by an adenine-deficient mutant is suppressed by mutation at other loci concerned with adenine synthesis (173). Recovery from inability to ferment lactose can arise by suppressor mutation in *Escherichia coli* K-12 (137), where its basis can be established by crossing. It is clear that the occurrence of suppressor mutations must be taken into consideration in interpreting mutation-rate studies.

There is some evidence that alleles exist in *Neurospora* that are similar in their heterocatalytic expression (inositol requirement) but range from extreme mutability to extreme stability (82). In asexual forms such as imperfect fungi and bacteria, precise determination of the physical basis of variation is difficult. However, indirect tests for allelism of genes with similar phenotypic effects are possible in exceptional cases. In bacteria, syntrophic crossfeeding has been used as a criterion of nonallelism with different biochemical blocks supposedly due to mutation at different loci (43). Similarly, heterocaryon tests for allelism have been applied in *Penicillium* (204).

The production of specific heritable alterations in bacterial cells by preparations containing desoxyribonucleic acid [*Pneumococcus* (161), *E. coli* (21) *Shigella* (249) and *Hemophilus* (2a)] could be considered as due either to transfer from one cell to another of a self-reproducing entity or to production of a specific gene-mutation by the transforming principle. The phenomenon is not limited to polysaccharide specificity, but other phenotypes have recently proved to be subject to transformation in *Pneumococcus*. An ex-

treme-rough variant may be transformed to rough under the influence of desoxyribonucleic acid either from rough or from smooth cultures (241). Demonstration of a reciprocal transformation from rough to extreme-rough (242) is significant, especially since the transformed cells are at a selective disadvantage, in contrast to all cases known previously. A specific protein can be acquired by *Pneumococcus* through transformation (12). Although, as a result of Boivin's untimely death, no new evidence is available, the apparent transformation of *E. coli* with respect to sucrose fermentation should be recalled (21).

The number of different characters being studied genetically in other asexual forms is constantly being extended [surveyed for bacteria in (141)]. Mutations have been obtained in bacteria that result in resistance to antibiotics (49), including aureomycin (51). Mutation leads to antibiotic production by actinomycetes (122), and impairment of polyene synthesis in the yeast *Rhodotorula* (26). The nature of thermophily is being investigated, and evidence has been obtained suggesting that ability to grow at high temperatures involves differences in the rate of protein synthesis and can be gained or lost by mutation in cultures of a number of different bacteria (3).

*Recovery of mutants.*—It is fairly simple to detect and isolate mutants with increased ability to metabolize particular carbohydrates, to synthesize essential nutrilites, to utilize specific nitrogen sources, or to resist inhibitors. The examination of large populations of microorganisms for rare biochemical mutants that cannot be directly screened has been considerably more tedious. However, a number of techniques have been developed to make this less laborious.

These methods [listed in (236)] include penicillin enrichment (41, 43, 199) and limited growth-factor enrichment [e.g. (42)] of mutant bacteria. With fungi, mutants with decreased synthetic abilities have been concentrated by filtration [e.g. (167)] and metabolic depletion (72) or obtained by visual selection on synthetic media. Development of plating techniques for *Neurospora* depend on restricting mycelial growth, by limiting inositol (239), or by adding sorbose (238). Substances such as griseofulvin (28) may prove useful in this regard.

## Gene Mutation

The problem of the nature of the gene and of gene-action can be approached either through analyzing gene expression or examining gene mutation and factors modifying it. Microorganisms are being used extensively to investigate delayed expression of mutations, the action of radiation, the relation between killing and mutation, the reversal of ultraviolet effects by visible light (photoreactivation), and the qualitative and quantitative effects of different mutagenic agents.

Discrepancies between the results of two alternate methods of calculating mutation rate in bacteria have been shown to arise from a delay in the

phenotypic expression of mutations (182, 186). Phenotypic [or phenomic (41)] lag between the time of occurrence of mutation and its consequent expression is characteristic of a number (52, 182, 186) but not of all (184) spontaneous and induced mutations scrutinized thus far in bacteria. The phenomenon has been demonstrated for mutants produced by radiation (52, 186) and chemical treatment (53) and indicated for mutations induced by use of radioactive phosphorus (211). Mutations resulting in gain as well as loss of synthetic abilities exhibit a delay in expression (43). In a preliminary report, the effect of ribonucleic acid in increasing the number of spontaneous virus-resistant mutants in nondividing cultures of *E. coli* is attributed to the abolition of phenomic lag (256).

*Genetic effects of radiation.*—Many recent investigations on radiation effects have made use of microorganisms (7, 70, 120, 134, 160, 191, 194, 201, 232, 235, 248, 259). A useful compilation of older data on dosage-effect relations is now available (265). The interpretation of dose-effect curves has been critically reexamined (8, 192, 193).

The problem of how to interpret killing (or sterilization) by mutagenic agents, including radiation, is difficult. That killing is primarily genetic and due to lethal mutation is indicated in x-irradiation of yeast, where the dose-survival curves of haploid and diploid cultures are of the form expected if killing is the result of single-events (exponential), and of multiple events (sigmoid), respectively (134). However, data on ultraviolet killing of multinucleate conidia of *Neurospora* indicate that at high doses, some mechanism other than lethal mutation is important (7). While the number of events required for killing by radiation generally shows a correspondence to the number of nuclei or chromosome complements present in each cell, it appears that effects other than mutation must be taken into consideration. Differences in the number of events necessary for mutation and for killing (52, 120, 232) by the same or by different types of radiation (70, 254) may provide clues as to differences in mechanism.

The nature of the mutational change that renders *E. coli* resistant to the lethal effects of x-rays, ultraviolet, and nitrogen mustard, without changing the frequency of mutation to phage-resistance (29, 52, 254), is still obscure. A possible basis for increased resistance may be that the resistant strain, B/r, synthesizes several times as much desoxyribonucleic acid as does the sensitive strain, B (178). The two strains differ in that the lethal effects of ultraviolet are reversed by heat only in strain B (5).

Whether strain B/r is similar to B with respect to rate of occurrence of mutations other than those to virus-resistance (e.g., mutations affecting growth-factor synthesis) has not been determined, and it is not known whether phage-resistance in strains B and B/r is recessive to susceptibility, as is the case for *E. coli* strain K-12 (140). Information of this type might indicate whether increased nucleic acid content is associated with changes in ploidy, or whether resistance to killing involves a change not affecting the genotype as a whole. If susceptibility to killing and mutation are sub-

ject to independent alteration, one might also expect to find cases in which a decrease in mutability occurs without any increase in resistance to the lethal effect of mutagens. Although no such examples have been reported in microorganisms, such a difference is suggested by comparing irradiation effects in the flies *Sciara* and *Drosophila* (38). Genetic differences in susceptibility to x-ray injury not affecting mutability have been reported in a higher plant (223).

The phenomenon of photoreactivation is likely to be important for analysis of the precise mechanism of ultraviolet mutagenesis and is potentially a valuable tool in examining gene structure and properties. If killing and mutation are differentially reversed by visible light, the two effects of ultraviolet radiation can be studied independently. The lethal effect of ultraviolet irradiation on *Streptomyces griseus* conidia can be strikingly reversed (400,000 fold) by subsequent exposure to visible light (123). Photoreactivation has also been demonstrated in *E. coli* for killing and for mutation to virus resistance (124, 187), in bacteriophage (59), *Neurospora* (86), *Penicillium*, *Saccharomyces* (124), and *Paramecium* (125) for killing, and in *Arbacia* eggs for inhibition of cleavage (20, 168). The proportion of cells reactivated by visible light is independent of the ultraviolet dose [(124, 187) dose reduction principle], and the mutation rate in *E. coli* is apparently reduced by the same dose-independent factor as is killing (187). Reactivation is possible only in a relatively short time following ultraviolet exposure, and only wavelengths below 5,100 Å are effective, with a maximum at 3,650 Å for bacteriophage (59a). X-ray effects are either not reversible or very slightly so (59a). In strain B of *E. coli*, heat is effective in reactivating ultraviolet irradiated cells (5), and in *E. coli* K-12, substances with peroxidase activity (catalase, peroxidase, and ferrous sulfate) will reverse the lethal effect (177).

Several intriguing problems are raised by the photoreversal phenomenon. If the primary effect of ultraviolet is due to lethal mutation resulting from specific absorption by nucleoprotein, how is reversal accomplished by visible light, which is absorbed only by other cellular components? In contrast, if killing and mutation are both secondary effects of ultraviolet, perhaps due to organic peroxide formation, does visible light act by photodecomposition of the peroxide, or by interrupting the chain of events culminating in mutagenesis? Finally, why is a constant proportion of ultraviolet induced events reversed by visible light?

*Chemical mutagenesis.*—An intensive search for mutagenic chemicals, stimulated by the pioneer work with mustard gas, has made use of a variety of microorganisms and a number of types of mutations as indices of activity. Some of the most precise quantitative data on mutation rate in microorganisms and on the effect of various agents on mutation have been obtained for mutation of *E. coli* from virus-sensitivity to resistance (30, 255), while the production of biochemical mutants has been investigated in bacteria, *Neurospora*, *Penicillium*, and other microorganisms [see (10)].

Although directed mutation, if it occurs, would seem most likely with

specific chemical mutagens, there has been no convincing evidence that different types of mutants are obtained with chemical treatment than with irradiation, or that specific chemical mutagens have a differential effect on specific genes [for a possible exception in the case of phenol-induced mutations in *Drosophila*, see (96)]. The types of biochemical mutants in *Neurospora* obtained following treatment with hydrogen peroxide and with hydrogen cyanide are not significantly different from those obtained with ultraviolet (248). Caffeine and other methyl-xanthines will produce biochemical mutants of *Ophiostoma* similar in type and frequency to those induced by radiation (74).

Back-mutation of a given gene from nutritional deficiency to independence is technically easier to use as a measure of mutagenic effectiveness than is mutation to dependence for growth factors. In *Neurospora*, x-rays, ultraviolet, nitrogen mustard, or $P^{32}$ increase the reversion frequency of an inositoless gene (84). Back-mutation of another specific gene (adenineless) in *N. crassa* has been used to evaluate mutagenic action, with positive results in the case of x- and ultraviolet radiation, nitrogen mustard (128), diazomethane (116), and organic peroxides (54). No activity could be demonstrated for phenol and urethane (116) although they have been reported to be mutagenic (30, 96).

A possible role of peroxides in ultraviolet-induced mutation was first suggested by the mutagenic activity of irradiated media (232a). Peroxide pretreatment of media was shown to increase the rate of mutation of *Staphylococcus* to penicillin and streptomycin resistance (259, 260) and of *E. coli* to phage resistance (94). Irradiated medium, peroxide, and hydrogen cyanide are all reported to produce biochemically deficient types in *Neurospora* (248). A number of organic peroxides are even more active than hydrogen peroxide (54) as judged by back-mutation rate in *Neurospora*. The general mutagenic activity of peroxide in microorganisms seems firmly established.

An important role of peroxides in ultraviolet mutagenesis is hard to accept in view of the specific action-spectrum for mutation by ultraviolet light which indicates absorption by nucleic acid (33). [See (160) for a possible exception]. Peroxide mutagenesis could, perhaps, be reconciled with the fact that the effective ultraviolet is absorbed by purines and pyrimidines if the peroxides responsible for mutation arise predominantly as the result of ultraviolet absorption by nucleoprotein (probably adjoining or making up the gene itself).

Although a number of chemical agents with marked affinity for nucleoprotein have been found to increase the frequency of mutation to virus resistance in *E. coli*, not all chemicals which might be expected to have this affinity are mutagenic (255). Similarly, a number of carcinogens, though not all, are mutagenic for *E. coli* and other organisms, and some noncarcinogenic chemicals are also mutagenic (10, 50, 133).

Although the same spectrum of mutants is apparently obtained in a given organism with a variety of treatments, the spectra may differ from one or-

ganism to another. Such differences in mutation pattern as have appeared could be the result of intrinsic physiological or genetic differences or of different isolation and screening techniques employed. The discrepancies observed with different chemical mutagens, especially relatively weak ones, and with different organisms are perhaps attributable to differences in absorption, penetration, and metabolism of the agent. Mutation may be the result of metabolic products of the substance tested, as suggested by observations that formalin acts as a mutagen in *Drosophila* only after it has reacted with ingested food (11), and that hydrogen peroxide treated media are mutagenic for bacteria whereas hydrogen peroxide is not (259). The number of mutations recovered in *Neurospora* following nitrogen mustard treatment varies considerably with pH, temperature, pressure (165), age and condition of the spores, and related physiological factors (172). Similar variations occur with *Achromobacter* (171).

Current views of the mechanism of mutagenesis involve two different concepts. One is that energy liberated directly by radiation and highly reactive chemicals raises the energy state of the gene molecule and thereby increases gene-mutation nonspecifically. The mutagenic effects of visible light on cells in the presence of photosensitizing agents (120) may be due to such action. On the other hand, mutagens might specifically interfere with the metabolic processes essential for normal gene reduplication and, hence, result in gene mutation. This would seem especially likely for the energetically less active mutagens [antimetabolites (74), chemicals with nucleic acid affinities (255), and possibly antibodies (61)].

## Genetic Change in Populations

Persistent changes affecting adaptive characters in microbial populations were long interpreted as resulting directly from the action of a changed environment on the hereditary constitution of members of the population, i.e., to directed mutation or transformation; this is in marked contrast to the fact that directive action of the environment has never been observed to evoke specific mutations in macroorganisms. The inconsistency has been resolved by demonstrating that inherited changes in microorganisms, as in other forms, originate as random events (mutations) in individuals within a population, and that the directive role of the environment is limited to the selection of variants. Criteria for distinguishing between random or directed origin of mutants in populations of clonally reproducing organisms were developed and applied by Luria & Delbrück in 1943 (155). In their original studies and in numerous cases analyzed since [(63) listed in (183)], it has been possible to demonstrate that variants arise by random mutation, independently of the selective environment.

Methods for determining mutation rates in clonal systems have been critically evaluated (136, 182), applied in a number of instances, and developed for mutations to growth-factor dependence (147). Alternate methods of calculating mutation rate have contributed to the study of delayed pheno-

typic expression of newly arisen mutant characters. Repeated demonstration of mutation and selection as the mechanism of adaptive change in microbial populations makes it imperative to distinguish between this and a physiological system where adaptation occurs in direct response to novel conditions. There can be little value in an analysis of the kinetics of adaptation that fails to distinguish between physiological adaptation and mutation followed by selection (103, 104). The inadequacy of such investigations has been pointed out (153, 174, 183).

Recognition of the role and importance of selection within heterogeneous populations has led to detailed studies of competition and population equilibria in bacteria (53, 213 to 218, 254). Some mutants with decreased synthetic abilities are at a selective advantage in certain environments (4, 72, 73, 127, 213, 257). Similar observations have been made in the case of a mixed population of nuclei within the same cytoplasm in a heterocaryon (212). In one instance, nuclei possessing two independent mutations impairing synthesis of the same essential metabolite are able to outgrow and replace nuclei with only one of these mutations (173).

## Mechanism of Transmission and Genetic Recombination

*Advantages of sexual organisms.*—Unless one can test for segregation and recombination by making sexual crosses, it is difficult or impossible to determine whether the inherited change is chromosomal or extrachromosomal and whether it is due to quantitative chromosomal change, to chromosome rearrangement, or to point-mutation. All these types of genetic changes can result in altered phenotypes. Knowledge of the type of change that has occurred becomes especially important for interpreting the mechanism of action of mutagenic agents, but has often been ignored in studies on mutagenesis in microorganisms.

Aside from making possible an analysis of the basis of inherited differences, segregation from a sexual zygote facilitates the building up of desired gene combinations, provides criteria of allelism, makes possible the construction of genetic maps summarizing recombinational behavior, and facilitates the correlation of genetical with cytological observations. Many sexual microorganisms possess features especially favorable for genetic analysis—the predominance of haploidy, survival of all four products of a single meiosis, the occurrence of heterocaryosis, and the capacity of multiplication under rigidly controlled environmental conditions.

*Formal genetics of fungi.*—The ascomycete *Neurospora crassa* has been most extensively studied in this respect. Extensive linkage data, accumulated chiefly since 1941, have been summarized (13, 110). Yeast genetics has reached the stage where sufficient data for construction of genetic maps are available (150, 151). Earlier work relied exclusively on genetic differences occurring in nature; stable mutants requiring growth factors have now been induced (203, 208) and found useful as genetic markers (202, 203). The puzzling phenomenon of irregular segregations giving ratios other than one:one for

a single pair of alleles, attributed to gene-to-gene transfer (149, 151), has been observed independently, and evidence has been presented for the occurrence of supernumerary nuclear divisions in the ascus (253). Such behavior would make irregular segregation understandable in conventional terms without invocation of any new principle. Delayed (long term) adaptation of yeast to galactose utilization appears to result from mutation followed by selection (179), rather than from a mechanism of slow adaptive enzyme formation, as proposed previously in a similar instance (252).

Both with *Neurospora* and *Saccharomyces*, formal genetic studies have been greatly facilitated by the use of mutations affecting synthesis of specific growth-factors or utilization of specific substrates. These characters have the advantage of being unambiguous and easily identified [see (197, 206) regarding auxanographic techniques], and mutants can be screened on selective media. Now that growth-factor mutants have been obtained in the mushroom *Coprinus fimitarius* (71), the smut-fungus *Ustilago maydis* (197), the ascomycete *Glomerella* (167), and the phycomycete *Allomyces* (263), study of these fungi should be facilitated. The use of growth-factor differences as markers has for the first time made genetic mapping possible in a homothallic organism, the ascomycete, *Aspergillus nidulans* (1, 205).

*Recombination in bacteria and virus.*—Genetic recombination in bacteria has been independently confirmed and further studied by workers in at least five laboratories (34, 36, 95, 119, 175, 185). There is evidence that the phenomenon is not confined to the single strain of *E. coli* in which it was originally demonstrated (34). In one stock, it is possible to obtain heterozygous diploids, permitting a study of dominance relationships and making possible the recovery of segregants under nonselective conditions (140). Recombination types, previously selected on the basis of growth-factor characters, have now been selected using inhibitors (142).

Genetic recombination in *E. coli* bacteriophage (156) has been further studied for differences occurring within type T-2 (101) and between different members of the T-series (154). The nonrandomness of recombination between certain factors suggests linkage.

*Cytogenetics.*—In contrast to the numerous features of sexual microorganisms that favor genetic analysis, they have been considered singularly poor material for studies of chromosome cytology, and correlated cytological and genetic studies have been rare. [Certain protozoa constitute a striking exception, e.g. (37)]. It now appears that the difficulties can be overcome in many cases. *Neurospora* has been the most thoroughly studied of the fungi (55, 68), and in *N. crassa*, where chromosome number was not even certain by old techniques, chromosomes can now be morphologically distinguished from one another and translocations recognized (162, 220).

Cytological observations in ascomycetes (105a, 190) agree with all genetic evidence in opposing the theory of brachymeiosis (an extra reduction division following two successive nuclear fusions); chromosome number is reduced to haploid during the first meiotic division, and segregation of

alleles is complete after the second division in every instance that has been carefully studied. Use has been made of smear techniques to demonstrate that the life-cycle of certain species of the phycomycete *Allomyces* is predominantly diploid (60), and to correlate specific chromosomes with occurrence of different sex types in the ascomycete *Hypomyces solani* (105).

The refined methods by which Robinow succeeded in differentially staining bacterial chromatin (210) have been found useful in a wide variety of bacteria [e.g. (117)], for the observation of nuclei in fungus spores (8, 13), and for the demonstration of chromatin bodies in *Rickettsia* (209). Observations of chromatin bodies in bacteria have also been made using the electron (102) and phase-contrast (244) microscopes. Bacterial cytology has been reviewed (126).

## Biochemical Genetics

During the past several years increasing numbers of microorganisms have been examined for the production of mutant biochemical characters [see (197, 239)]. In general the mutants are characterized by relatively simple requirements for essential nutrilites analogous to the types originally described in *Neurospora;* a few instances of somewhat more complex nutritional requirements have been described. There is considerable new information on the mechanism of biosynthesis of certain essential nutrilites, and the techniques of biochemical genetics have been extended to other biological phenomena such as photosynthesis and luminescence, nitrogen fixation, and carbohydrate metabolism.

Certain heterotrophic mutants in *Chlorella* are defective in carbon dioxide fixation, others in oxygen evolution (46). These are unable to live photosynthetically although they contain apparently normal chlorophyll (45). Chlorophyll synthesis has also been studied by means of *Chlorella* mutants; protoporphyrin, magnesium vinyl pheoporphyrin, and pheoporphyrin $A_5$ are accumulated by different mutant strains (91, 92, 93). In *Chlorella,* as in *Euglena* (159, 207) and *Scenedesmus* (108), it is difficult to establish the genetic basis of these inherited differences. However, mutants with impaired photosynthetic abilities have now been obtained in *Chlamydomonas* (145), a sexual form where combined genetic and biochemical investigation should permit a distinction between genic and extranuclear modification.

The production of light by living organisms may be attacked by analogous techniques. Biochemical mutants have been obtained in luminous bacteria (164). So far, no strains have been obtained in which a nutrilite is required specifically for luminescence. Another promising field has been opened with the isolation of mutants of *Azotobacter* which are unable to fix nitrogen (261).

*Carbohydrate utilization.*—The development of ability to utilize as carbon sources substances not normally used has been explored considerably. New capacities may be gained by physiological adaptation, by mutation, or by enzyme adaptation superimposed on mutation (130, 174, 176).

One of the difficulties in analyzing the effect of mutation on the utilization of the rarer hexoses, such as galactose, or of disaccharides, such as maltose or lactose, is our incomplete understanding of the normal metabolism of these substances. Contributions bearing on this include the demonstration of galactose-6-phosphate as an intermediate in galactose utilization (243) and the finding that an *E. coli* mutant, unable to use glucose as carbon source, can nevertheless grow on maltose, owing to the fact that disaccharide utilization proceeds via glucose-1-phosphate without free glucose serving as a normal intermediate (57). Phosphate metabolism *per se* may be affected by mutation in *Neurospora* (112).

The occurrence of *Neurospora* mutants which require succinic acid or related substances for growth (146) was interpreted to mean that the Krebs cycle probably functions in *Neurospora* and that the mutants are defective in the synthesis of succinic acid. Mutant strains of *Neurospora* have been obtained which specifically require fatty acids for growth (143). The higher unsaturated fatty acids are apparently derived from the corresponding saturated fatty acids. One mutant is able to utilize either acetate or several of the higher fatty acids (144). Mutants requiring acetate have been obtained in *Neurospora* (237) and *Azotobacter* (121), and a strain of this type has been used as an experimental tool for the examination of sterol synthesis in *Neurospora* (196). Ergosterol, which has been identified as the major sterol in this organism (195), is synthesized from acetate or related compounds, and ergosterol synthesis in *Neurospora* seems analogous to that of cholesterol in the rat (19).

*Nutrilite biosynthesis.*—The use of biochemical mutants of microorganisms to elucidate mechanisms of amino acid biosynthesis has recently been reviewed (236) emphasizing similarities in the biochemical mechanisms of mammals and microorganisms. Additional data are available on sulfur metabolism in *Aspergillus*, *Penicillium*, and *E. coli* (106, 107, 219) and on the role of *p*-aminobenzoic acid in methionine synthesis (132, 234). A new type of mutant in *Neurospora* involving a deficiency in ammonia utilization has been investigated (69); this probably results from a genetic block in amination of $\alpha$-ketoglutaric acid.

New information has been reported on tryptophane metabolism in microorganisms and on the mechanism of conversion of tryptophane to nicotinic acid. The synthesis of tryptophane from anthranilic acid involves loss of the carboxyl group (189). The operation of a metabolic cycle involving the production of anthranilic acid from tryptophane and kynurenine has been suggested (98), but there is contradictory evidence (25). The close metabolic relationship of quinolinic acid to hydroxyanthranilic acid and to nicotinic acid has been demonstrated in *Neurospora* (24) and in the rat (99), and kynurenine has been established as an intermediate in nicotinic acid synthesis in *Neurospora* through its isolation as the acetyl derivative (262).

It has been reported that tryptophane can be replaced for a *Neurospora* mutant by the aromatic compounds phenylalanine or tyrosine and to some

extent by *trans*-cinnamic acid (188). Interconversion of these compounds is somewhat difficult to picture, since another type of mutant obtained in *E. coli* (43) and in *Neurospora* (239) has a multiple requirement for the four aromatic compounds, phenylalanine, tyrosine, tryptophane, and *p*-aminobenzoic acid, but responds to a single compound, shikimic acid, which is a probable intermediate in the oxidation of aromatic compounds by bacteria (230). In these mutants the four required aromatic compounds all seem to be derivable from shikimic acid, and there is no evidence for their interconvertibility.

A new suggestion regarding the synthesis of aliphatic amino acids has been provided by the isolation and identification of $\alpha$-$\beta$-dihydroxy-$\beta$-methyl butyric acid from a mutant strain of *Neurospora* requiring isoleucine and valine (2).

*Gene-enzyme relations.*—The hypothesis that genes control specific biochemical reactions by determining enzyme specificity has been examined in a number of different ways. Information has been obtained as to the effect of gene mutation on the production and activity of several specific enzymes. Investigations in animals and higher plants have led to the view that mutations which result in inability to carry out a particular reaction entail the complete absence or complete inactivity of the specific enzyme molecule concerned (14, 113). Yet mutation cannot invariably involve gene loss, as indicated by the existence of multiple-allelic series (85), or enzyme inactivation, as evidenced by mutants in which biosyntheses are either impaired (172a), or sensitive to changes in pH (233) and temperature, or by mutants in which enzyme stability is decreased (158).

A few such relationships have been critically examined in microorganisms. Adenine deaminase from a temperature-sensitive adenineless strain of *Neurospora* failed to show *in vitro* any significant difference from the wild-type enzyme in its temperature characteristics (166). (This enzyme may, however, not be the one associated with the genetic change.) *Neurospora* enzymes involved in pantothenic acid synthesis have also been investigated (246, 247); the requirement of a pantothenicless mutant is apparently not due to complete absence of the critical enzyme, but results, rather, from failure of the enzyme to function *in vivo* since active enzyme preparations were obtained from the mutant strain. A third enzyme system became available for investigation when tryptophane synthesis was shown to be carried out *in vitro* by an enzyme from wild-type *Neurospora* (245). A mutant strain which, from its biochemical specificity, should not be able to synthesize tryptophane from indole and serine, nevertheless contains an enzyme that can carry out this step *in vitro* (88). The enzyme from the mutant is clearly inhibited by components of wild-type or of mutant mycelium, and becomes active only when freed from this inhibitors. If these examples are representative of growth-factor mutants generally, gene mutation results in absence of an enzyme less frequently than previously suspected and may entail modification or inhibition, rather than loss, of a particular enzyme.

Further information on gene-action has been obtained by studying complex phenotypic effects of single mutations. Numerous examples have been reported in *Neurospora* and other microorganisms in which biochemically deficient mutant strains are subject to specific inhibitions of growth and metabolism in contrast to relative or complete absence of inhibition on the part of the wild-type. [See (236) for review]. Although a purine-deficient strain of *Neurospora* can evidently convert adenine to guanine, excess guanine inhibits adenine utilization and growth (67). Complex metabolic interrelations have also been demonstrated between pyrimidines, lysine, and arginine (112a). Inhibitions in microorganisms may also be brought about by natural metabolites (40), by unnatural metabolites such as D-serine (44), by foreign metabolites such as canavanine (109) or by antibiotics. The relation of some of these inhibitions to growth-factor synthesis has been shown in several instances.

Glutamic acid dependence in *Staphylococcus* is associated with penicillin sensitivity, and mutation to amino-acid independence confers resistance to penicillin due to the effect of penicillin on glutamic acid assimilation (75, 76). In *Salmonella*, a cysteine requiring mutant is sensitive to penicillin, whereas the wild-type organism is resistant (200).

Even natural amino acids may affect amino acid assimilation and thereby inhibit growth, as shown for a lysine dependent strain of *Streptococcus faecalis* in which the assimilation of lysine is inhibited by arginine plus histidine (181). Arginine has previously been shown to be inhibitory for a lysineless strain of *Neurospora* (56). Although this and similar inhibitions in *Neurospora* have been thought to involve intracellular assimilation of the required amino acid, interference with passage across the cell wall may be responsible in some cases, such as that described for *Streptococcus*.

Still other examples of the complexity of interrelations in living protoplasm are the remarkable instances in which requirements for particular antibiotics are acquired by mutation [cf. (263a)]. Investigations on a sulfanilamide-requiring mutant strain of *Neurospora* seem to provide a logical basis for interpreting the development of such peculiar requirements. This strain apparently synthesizes a sufficient excess of *p*-aminobenzoic acid to inhibit itself unless sulfanilamide is furnished to antagonize the normal metabolite (264).

In other instances, inhibitions may be due to accumulation of normal intermediates as a result of genetically blocked reactions or to the production of inhibitory metabolites from these intermediates. Derangements of this sort are known in the accumulation of a purple pigment in adenine dependent mutants of yeast (208) and of *Neurospora* (173), and in the production of a yellow pigment from *p*-aminobenzoic acid by strains of mycobacteria (170) and *Neurospora* (39). Accumulation of inhibitory intermediates has been invoked to explain the requirement of a *Neurospora* mutant for isoleucine and valine (22).

All these examples make it obvious that analysis of gene-enzyme relations

is complicated by interactions, inhibitions, and accumulation of intermediates, which may result in what appear to be multiple effects of the mutation of a single gene.

That changes in single enzymes are relatable to differences in single genes has proved fruitful as a working hypothesis. However, it is questionable whether one gene controls the activity of only one enzyme and whether the specificity of one species of enzyme is attributable to but one gene. In *E. coli* K-12, several allelic lactose-nonfermenting mutants differ from one another in ability to use closely related glucosides (139, 141). Whether pleiotropism is in this case due to a direct effect on configuration of the enzymes concerned or to some indirect action affecting adaptive production of the enzymes, is not known. Several reports indicate that more than one gene may be concerned in determining the activity of a particular enzyme. A number of nonallelic genes are known to affect the production of tyrosinase in *Glomerella* (167), and of lactase in *Neurospora* (23) and *E. coli* (139). If utilization of the substrates is shown to involve only a single enzyme in each case, more than one gene must be concerned with the specific activity of that enzyme. The enzymatic basis of restored growth-factor synthesis resulting from suppressor mutations has not been investigated.

It seems clear that a single enzyme generally has only one function. Tyrosinase is a possible exception. Single mutations in *Glomerella* differ from one another in the relative extent to which tyrosinase activity is changed on the two substrates, tyrosine and dioxyphenylalanine (167). Mutation in a case such as this may involve modification of specific enzyme structure such that different catalytic functions of the single enzyme are altered independently of one another, perhaps by modifying to different degrees the affinity for the two substrates.

It is possible that lack of enzyme activity *in vivo* or in impure preparations can be due to specific inhibiting factors rather than to alteration of specific enzymes (246, 247). Interpretation of cases such as those cited above should be made with caution until a critical demonstration of enzyme alterations has been accomplished *in vitro* using purified enzyme preparations.

## Genetics and Evolution

The ability to recognize and distinguish mutation, selection, and random fluctuation in microbial populations and to analyze population changes in terms of these factors makes possible an extension to microorganisms of concepts developed in connection with the evolutionary genetics of higher forms by Fisher (69a), Wright (258a), Dobzhansky (57a), and others. Such features of microorganisms as rapid multiplication, haploidy, heterocaryosis, and multiple sex systems should provide opportunities for testing the tenets of population genetics under conditions especially favorable for experimentation [see (138, 153, 204, 213)].

The evolutionary significance of sexual recombination and of mechanisms promoting outbreeding in heterothallic microorganisms has been discussed,

extensive data have been compiled on breeding systems in fungi, and hypotheses have been developed regarding the selective value of multiple loci and multiple alleles governing compatibility (169, 250, 251).

In conclusion, studies in the genetics of microorganisms are contributing significantly both to a fundamental understanding of evolutionary theory (the mechanics and dynamics of change) and to an understanding of the precise manner in which genetic mechanisms govern cellular processes. Comparative genetics, like comparative biochemistry and comparative physiology, deals with fundamental biological phenomena that are common to many diverse organisms. A continuation of the present rapid development in this area can be expected to add increasingly to our understanding of all forms of life.

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# GENETICS OF VIRUSES[1]

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Capacity for variation was recognized as an attribute of the viruses early in their investigation. The classical example of the type of investigation that has contributed the bulk of knowledge concerning variation of viruses, especially those attacking animals, is the modification by Pasteur of the "street virus" of rabies to the "fixed virus" of the laboratory. This was accomplished by passage through a series of rabbits, during which certain changes in the biological properties of the virus took place. Such modifications, brought about by adaptation to new host tissues, appear to be entirely analogous to the genetic changes observed and studied more extensively in higher forms, and of late they have been described mainly in those terms. It should be pointed out, however, that when variation of a virus is observed, what is actually seen is variation in a large population of virus particles. This qualification should be in mind when terms are used, such as "mutant," that were employed originally in describing individuals.

Taxonomically speaking, viruses fall into natural groups on the basis of their physical and chemical nature, the type of disease produced, and their immunologic character. It appears entirely justified to assume that the naturally occurring groups, as well as the modified laboratory strains, arose through genetic variation and selection, in a manner similar to that ascribed to more highly organized life. It is significant in this connection that the current edition of Bergey's Manual (1) for the first time contains a classification of the viruses.

The present review, with no attempt to be exhaustive, will first discuss some group relationships, as well as specific observations on variation in certain groups. An attempt will then be made to correlate the data by discussing some of the mechanisms presumed to underlie such virus variation.

Findlay (2) has thoroughly reviewed the subject of variation in animal viruses up to 1939, and only small portions of the work covered by his reviews will be repeated here. The stimulating lectures by Burnet (3) have contributed greatly to thoughts on variation and evolution of viruses. Genetics of plant viruses, because of the recent review by Kunkel (4) as well as limitation of space and the writer's field of interest, will be mentioned only incidentally in discussion. The bacterial viruses are properly discussed elsewhere.

## The Arthropod-Borne Encephalitides

A group of neurotropic viruses that has emerged in recent years as a definite "family" has been discussed by Hammon & Reeves (5) and by Hammon

[1] This review covers the period from 1939 through 1949 with occasional references to earlier work.

(6). This group includes the viruses of Eastern, Western, and Venezuelan equine encephalomyelitis, St. Louis, Japanese B, and Russian spring-summer encephalitis, and louping ill. Less complete evidence indicates that a number of others, such as the West Nile, Semliki Forest, Ilhéus and California viruses may also belong to the family. Mosquitoes, ticks, mites, and an assassin bug are implicated in transmission of one or more of these viruses, and there is reason to believe that all members of the family are arthropod-borne. Although no antigen common to the entire group has been demonstrated, immunologic relationships between several subgroups have been observed. Thus, St. Louis, Japanese B, and West Nile viruses have been shown repeatedly to be related when specific antiserums are used in neutralization and complement-fixation tests such as those performed by Casals (7). The two members of the group that are transmitted by ticks, louping ill and Russian spring-summer encephalitis, are closely related immunologically, as reported by Casals & Webster (8), and the California virus is reported to show slight relationship to St. Louis and Japanese B in the complement fixation test, according to Espana & Hammon (9). Eastern and Western equine encephalomyelitis viruses were shown by Howitt (10) to overlap antigenically.

Hammon (6) has used these facts as well as others to support the hypothesis that these viruses may have a common ancestry. In fact, he and his colleagues describe (11) what may be a "stem virus" for at least two of the closely related members of the group. From mites collected in the field, a virus, or mixture of viruses, (BFS-867) was recovered which, upon several passages through mice, was identified as St. Louis encephalitis virus, but when passed through embryonated eggs was antigenically identical to Western equine encephalomyelitis virus. An antigen, prepared from early mouse brain passages in appropriate dilution, fixed complement in the presence of both St. Louis and Western equine antisera. Various observations supplied evidence that a mixture of the two viruses could not have accounted for the observed results.

In support of his hypothesis, Hammon (6) has pointed out that infections with both St. Louis and Western equine viruses, have occurred repeatedly in fowls, horses, and humans in the same localities and at the same times. Both have been isolated from the same species of mosquito (*Culex tarsalis*) and chicken mite (*Dermanyssus gallinae*). Both viruses are approximately the same size and the two infections are similar pathologically and clinically. Antibodies against both viruses have been found to arise at the same time in human cases of encephalitis and such "double infections" apparently occur too frequently to be accounted for on the basis of chance alone.

If such a stem virus exists, with potentialities for becoming either St. Louis or Western equine virus, it is evident that infection of the human (and animal?) host can occur with the stem virus (to account for the large number of "double infections"), and the change in character toward St. Louis or Western equine that takes place after isolation, can be ascribed to a selective action of the medium chosen, i.e., mouse brain or chick embryo. Once the shift is made, however, it would appear to be stable because no evidence has

been brought to light that one virus type shifts to the other. For example, it has not been reported that culture of St. Louis virus in eggs changes it to an equine virus, nor passage of equine virus in mouse brain modifies it in the direction of the St. Louis type. Further, the postulation seems necessary that this shift of stem virus to the St. Louis or Western equine character may take place in nature since serological tests often indicate a "pure" infection with one virus or the other in both man and animals (12, 13). The evidence that Hammon has presented for a stem virus is extremely interesting and confirmation will be eagerly awaited.

In any case, this study illustrates a complexity of virus investigation that has been generally recognized only in recent years. It is common experience that a newly isolated virus multiplies poorly at first in the laboratory; few or many passages may be necessary before the strain is adapted to a laboratory host. It has been realized, of course, that this adaptation is in essence a variation phenomenon in which the virus acquires ability to multiply readily in the new host. What has not been generally realized is that other profound changes may occur at the same time, including a shift in antigenic character as illustrated by Hammon's experiments and by observations with influenza virus to be described later. It should be noted that Manninger & Lazlo (14) called attention in 1930 to antigenic instability of foot-and-mouth disease virus as it occurs in nature. They realized that the strains after stabilization by guinea pig passage were no longer the same entities that caused the natural disease.

## The Influenza Viruses

Intensive study of the influenza viruses has led to a number of observations regarding the genetics of this group. Beside the usual methods applicable to viruses for titration and for immunologic study, the capacity of these viruses to be adsorbed upon, agglutinate, and be eluted from erythrocytes, described and studied by Hirst (15) and others, has provided a simple, rapid *in vitro* technique for performing quantitative experiments including serologic tests, with these and certain other viruses. Burnet (3) has pointed out the great "mutability" of these viruses, as evidenced by diversity of strains in nature and by numerous examples of variation in the laboratory. The recent discovery of A′ strains (16), not previously encountered, may be due to the fact that they represent a recent variation.

*Antigenic variation.*—The main groups of influenza viruses have been distinguished mainly on the basis of complete, or almost complete, immunologic dissimilarity. These are: influenza A, B, and swine influenza. Within these groups, and especially in the case of influenza A, with which most of the work has been done, many strains have been found to be immunologically different, although all possess an antigen common to the group (17, 18, 19). Antigenic differences have been found even in strains from a single epidemic (20). The strains used for these early tests, performed by cross-neutralization and cross-immunization tests in mice, were necessarily all mouse-adapted and the question was voiced at that time (18) whether the adapta-

tion process might alter the antigenic character of a strain. This question was not satisfactorily answered until improved techniques were available. Hirst (21, 22) studied the immunologic relationship of epidemic strains that had been established and maintained only in eggs, and employed the hemagglutination-inhibition test (inhibition of viral hemagglutination by specific antiserum). Contrary to the immunologic diversity found at the same time for older laboratory strains, he found a high degree of homogeneity among strains isolated from persons in widely separated localities. To study more directly the question of whether antigenic variation occurs with adaptation to animals, Hirst (23) established two strains of influenza A in eggs directly from patients and later, beginning with another sample of original throat washings, established new lines of the same strains by passage first to ferrets (6 passages) and thence to mice (30 passages). Antisera against the four lines were then prepared and cross hemagglutination-inhibition tests performed. It was found that the egg lines were similar, but the mouse line in each case was different from the egg line of the same strain, and the two mouse lines differed from each other.

These results emphasize the point made in a preceding section, that adaptation to the laboratory host may defeat its own purpose, because the result may be a variant sufficiently changed from the original to be of questionable value for certain purposes. In the case of influenza virus, Hirst's results suggest that less change occurs with passage in the egg than in the mouse. It might be postulated that the more primitive tissue of the egg, or the undeveloped capacity for immune response, are factors in the apparently greater genetic stability of egg-cultured virus.

Antigenic variation has been observed also in strains maintained for longer periods in the laboratory. Francis (24) presented evidence that antigenic variation occurred between two lines of influenza A, strain PR8. He used one that had been transferred to tissue culture and then to eggs after the 70th mouse passage, and another that had been kept in mice for 593 mouse passages. The egg line had evidently changed its antigenic character early in its passages outside the mouse. Similar observations were reported by Sugg (25) who compared two lines of an influenza A′ virus. Antigenic difference was found between one line that had been maintained in eggs for 61 passages, and another that had been fully adapted to mice by 30 or more mouse passages beginning with the 38th egg passage. Starting with the egg line, passages through mice were again made. Adaptation was practically complete by the eighth passage as demonstrated by mouse mortality and extent of pulmonary lesions. The virus from these mice, however, still possessed the antigenic character of the egg line, indicating that the two types of variation, antigenic and mouse-adaptation, do not necessarily occur together.

Taylor reported (26) on the immunologic character of egg-isolated strains recovered at various times, and discussed the factors accounting for the great immunologic variability among influenza strains. He also mentions an experiment (27) in which passage of virus in the presence of immune serum resulted in a change in antigenic character.

*Variation in pathogenicity.*—The adaptation of strain WS of influenza A to brains of mice has been reported from two laboratories. Stuart-Harris (28) attributed his success to initiation of mouse passages with infected chick embryo brain, but Francis & Moore (29) were able to demonstrate neurotropism in the WS strain, and to a lesser extent in another, by direct inoculation of tissue culture material without passage through chick brain.

A number of studies have been reported on variants differing in their pathogenicity for rodent lung. Hirst (23) reported that two strains of egg-adapted virus produced no pulmonary lesions when first passed to mice intranasally. But titration of their lungs in eggs on the third or fourth day revealed as much virus as did similar titrations at a later date with the same strain after adaptation had occurred and pulmonary lesions were present. The suggestion from this result is that acquisition of pathogenicity depended upon some factor other than acquisition of ability to proliferate in the lung. Wang (30), in a similar type of experiment, used two lines of the same strain; they differed only in their pathogenicity for mouse lung. His observations were similar to those of Hirst (23), but he concluded that the difference was based upon the rate of multiplication in the mouse lung rather than the final amount of titratable virus present. In addition to growing more rapidly, the hemagglutination titer of the adapted virus, as contained in mouse lung, reached high values at 24 and 48 hr., while that of the unadapted line always remained at a low level. The question of a toxin such as the Henles (31, 32) and others have demonstrated comes up in this connection. Sugg (33) observed that an egg-adapted strain, although capable of only a slight degree of proliferation in first passages in mouse lung, nevertheless produced pulmonary lesions when sufficient quantities of the egg-cultured virus were introduced.

A correlation between pathogenicity and hemagglutinating ability, as noted above in the work of Wang (30), is seen also in a study reported by Friedewald & Hook (34). While investigating the susceptibility of hamsters to influenza virus these authors found that two strains of influenza A, when passed serially through hamsters, failed to produce lung lesions or death, and lung extracts were incapable of hemagglutination during the first six serial passages. In the seventh passage, however, severe lung lesions and death were observed, as well as increase in hemagglutinating and complement fixation titer. Although a change in amount of virus present, as determined by egg and mouse titration, is not clearcut, all the manifestations of virus activity at the seventh passage would seem to be due to greater growth at that point.

Some strains of influenza A have been carried by Burnet (35) through numerous egg passages until a point is reached where the pathogenicity for man is greatly reduced, but artificial exposure results in immunization. In some cases, further egg passage results in a loss of immunizing capacity as well (3).

*Variation in hemagglutinating properties.*—Burnet and his colleagues (36, 37) have described and studied a variation of influenza A, based upon differences in hemagglutinating ability for erythrocytes of different species, and

ability to proliferate in the allantoic cavity of embryonated eggs. When throat washings from influenza patients were injected into the amniotic cavity of the embryonated egg, the virus recovered in the amniotic fluid of the first generation was found to have very little hemagglutinating ability for chicken erythrocytes, but agglutinated guinea pig or human erythrocytes to relatively high titer. Second and subsequent passages in the egg, accomplished by transfer of amniotic fluid in low dilution, yielded an altered virus that was found to agglutinate fowl and guinea pig cells to approximately equal titer. The virus of the first passage is considered to be in the original (O) phase and was found to grow poorly or not at all in the allantoic cavity of the egg, and probably to be noninfective for the mouse lung. The virus could be kept in the O phase indefinitely by passage of high dilutions by the amniotic route. The altered virus, said to be in the derived (D) phase grew readily in the allantoic cavity and could be further adapted to the mouse lung. Intermediate phases were also described. As indicated by Burnet, this O→D change appears to be the result of selection of a spontaneous discontinuous variant (mutant); an estimate was made that a D phase particle appears about once for every $10^5$ to $10^6$ O phase particles. Once such a variant appears, however, it overgrows the O phase since the latter was found to multiply more slowly than the D phase in the amniotic cavity. No reversion from D to O was noted. Maintenance of O phase was accomplished by using as inoculum dilutions of amniotic fluid presumably great enough to reduce the content of D mutant below the point of probability of carrying over an infective dose of D.

Further studies were performed by Anderson & Burnet (38) with interepidemic strains, in contrast to epidemic strains used previously. These strains were also isolated in the O phase, but D phase appeared more slowly and intermediate phases possessed some characteristics of both O and D. The authors suggested that there may be a correlation between mutability, as expressed by rapid O→D variation and the ability to initiate an epidemic.

Burnet called attention to the possibility that the strains of virus naturally affecting man are all in the O phase, and that our established laboratory strains carried in the allantoic cavity (D phase) are not the same entities at all that occur in nature. Allantoic cavity cultures of some strains of influenza virus will infect man under artificial conditions, but whether such a virus has other properties of "natural" virus, such as high transmissibility, is unknown.

The ratio between hemagglutinating ability for fowl and guinea pig erythrocytes (F:GP ratio) has been used as the criterion of O→D phase variation. A low F:GP ratio, e.g., 1/10 or less, indicates O phase, while an F:GP ratio near unity is characteristic of the D phase. Subsequent investigations have confirmed the essential observations of Burnet and his group concerning O→D variation but have raised questions concerning their proper interpretation. Hirst (22) reported that he could not maintain virus in the O phase and had difficulty in distinguishing the phases. Magill & Sugg (39) found, as did Burnet's group, that virus derived from allantoic fluid was regularly in the D phase, and O phase was only present when amniotic passages (of

embryo lung and trachea) were made. However, they found it was not always possible to maintain a strain in the O phase. Quite unpredictably, some eggs of a given passage would contain O phase and others D phase. Reversion to O occurred when certain strains in D phase, including WS that had been through numerous ferret and mouse passages, were cultured in the amniotic cavity. Intermediate phases were also observed.

Further complexities appeared when it was found that the difference between O and D phase agglutination is subject to physical or chemical factors in the environment. Magill & Sugg (39) found that a shift to pH 5.6 of the suspending fluid resulted in a change to D type agglutination of allantoic fluids that had shown O agglutination. Briody (40) found that O phase virus can be induced to take on the properties of the D phase by means of heat, storage, or the addition of various inorganic ions to the preparation. Passage of such modified virus resulted in growth of O phase virus only. Briody further reported (41) that the shift of D in the direction of O phase results in a "pseudo-O" (XO) type that is similar to, but may be distinguished from, the true O phase. Friedewald & Hooker (34) also found that heat was effective in bringing out the agglutinins for chicken erythrocytes in extracts of infected lungs.

The hemagglutinating properties of influenza B strains also have been studied with respect to variation. In Burnet's laboratory (42) no discontinuous change was found with adaptation of influenza B to the chick embryo. Briody (43) found that he could define two phases of influenza B on the basis of F:GP ratio as in the case of O→D variation with influenza A. He postulates that the virus occurs naturally in an E (equivalent F:GP ratio) phase that can be maintained by appropriate passage techniques in the egg. This phase mutates to an H (high F:GP ratio) phase under certain conditions, and H phase can mutate back to E. Interference effects, influenced by the degree of dilution of the inoculum, are believed to be the controlling factor in the shifts.

In studying the rate of elution of virus from a red-cell-virus combination, Björkman & Horsfall (44) found that the PR8 strain (influenza A) characteristically showed a constant rate of elution, while the Lee strain (influenza B) is eluted more rapidly, with a large amount of the adsorbed virus coming off in the first 30 minutes. It was found that single exposure of these strains to lanthanum acetate or to ultra-violet light produced variants with a lowered rate of elution, the Lee strain after modification by such exposure being indistinguishable from the unmodified PR8 strain. Other properties were not changed, but the modified character persisted through a number of further passages. Additional experiments indicated that the modification may be related to the effect of salt concentration.

It is plain that the nature and significance of these changes in properties of influenza strains are not yet clear. Burnet (36) described the O→D change as a "discontinuous mutation" and regarded the D phase as a stable variant; others (22, 39, 41) have supplied evidence for considerable instability, including reversibility at least in part. Briody interprets the modifica-

tion of O phase to D by heat, etc., as a phenotypic variation (phenocopy) rather than genotypic, since the change was not inheritable. It appears that both types of variation may occur (mutation and phenocopy) and thus obscure the picture. With the demonstration that the "characteristic" F:GP ratios are so readily changed by environmental factors in the absence of virus multiplication, it is evident that the hemagglutination ratio is not an entirely satisfactory tool for studying subtle changes in the virus. It is apparent, also, that the results of viral hemagglutination tests are significant only when the conditions under which the test is made, e.g., nature of suspending medium and pH, are known and carefully controlled.

## The Pock Diseases

The pock diseases, including variola (smallpox), alastrim, and vaccinia of man, as well as a number of pock diseases of animals, were early recognized as constituting a family of related viruses. Findlay (2) reviewed and discussed theories concerning the evolution of this group, and recent work has not significantly altered the bases for those theories.

Ectromelia (mouse pox) has recently been added to the group on the basis of antigenic and other similarities to vaccinia, as reported by Fenner (45) and by Dickinson (46). Although vaccinia-like variants have been derived from some of the other animal pock diseases, Bennett, Horgan & Haseeb (47) have reported that immunologic relation to vaccinia is not always evident. The alteration of variola and alastrim to vaccinia has been discussed by Horgan (48) and by Findlay (2). In contrast to the transformation of variola virus alleged to occur with passage in rabbit skin, cultivation of this virus in the egg apparently is accompanied by no such change [cf. (49, 50)].

Cowpox is very similar to vaccinia, the one conferring complete immunity against the other, but Downie found that the two viruses could be distinguished on histopathologic (51) and immunologic (52) grounds. For the latter he used serologic cross tests with hyperimmune antiserums absorbed with elementary bodies. Two strains of each virus were used; the vaccinia strains had been passed in rabbits for years, but the cowpox strains were more recently adapted to rabbits. To what extent the differences noted may have been the result of variation during laboratory passages is not known. It is noteworthy that the two strains of cowpox appeared to be essentially identical, as did the two vaccinia strains.

Since several variants of vaccinia virus are recognized, e.g., neurovaccinia [cf. (2, 53)], the question has been raised whether different strains of vaccinia will all protect equally well against variola. Horgan & Haseeb (54) studied the immunologic relations among a group of vaccinia strains collected from a number of different localities and with different origins, e.g., from alastrim, variola, and neurovaccinia. Their results indicated that complete cross protection between the various strains occurred. Although of considerable value for practical purposes, this type of experiment is not designed to answer the question whether immunologic differences of perhaps minor character exist among the various vaccinia strains, as Downie reported in the case of

vaccinia and cowpox. Similar types of experiments to study the immunologic relations of variola, alastrim, and vaccinia, have been performed by a number of investigators using the monkey as the experimental animal. Horgan & Haseeb (55) review the work of others and report their own investigations in which infection of monkeys was induced by application of the virus to the scarified skin. The uncontrolled quantitative factor was recognized by the authors in their statement that solid immunity is dependent upon a strong local primary reaction. The necessity of using monkeys for this type of investigation restricts greatly the desired quantitation of the immunizing dose, the infecting dose, and the result, so that interpretation is difficult. Later work from the same source (56) led to the conclusion that a group of alastrim strains all had a basic antigen in common, but possessed minor antigenic differences. It is to be hoped that with the cultivation of these viruses in the embryonated egg, newer techniques will be applied to elucidate the immunologic pattern within the group.

## Miscellaneous Observations

*Rabies.*—Existing laboratory strains of rabies virus differ from one another in a number of characteristics of importance in selection of strains for rabies prophylaxis. Without discussing the problem and its investigations in detail, a few features will be pointed out here. A number of reports indicate that strains of rabies virus vary immunologically [cf. (57, 58, 59)], in virulence and infectivity, which can vary independently (60), and in ability to overcome immunity induced by vaccines. Wright & Habel (59) present evidence that this latter property is correlated with resistance to antibody in the neutralization test, but is not correlated with virulence, length of incubation period, or antigenicity. They point out that the six strains which they studied are all actually substrains of the original Paris strain fixed by Pasteur.

Several reports have appeared on adaptation of rabies virus to the embryonated egg [cf. (61)]. Koprowski & Cox (61) have described such a strain that was passed first through chick brain, and thence to chick embryo. Its potential value for immunization is seen in the fact that it seemed to have lost entirely its invasive power after intramuscular inoculation in the rabbit.

*Yellow fever.*—Established strains of yellow fever virus possess marked differences in virulence for various host species. The originally isolated (Asib) strain is highly pathogenic for rhesus monkeys (*Macaca mulatta*) as is true of most other African strains. In contrast, as Theiler has indicated (62), South American strains are generally less pathogenic for rhesus monkeys. Strains vary also in their degree of neurotropism, and this character has been enhanced at the expense of viscerotropism by passage through the brain of mice (63) and chicks (64). Apparently spontaneous reversion of such a strain to viscerotropism for the monkey was described by Findlay & MacCallum (65), and a similar reversion induced by extraneural passage in the monkey was reported by Findlay & Clarke (66). Immunologically, yellow fever virus is regarded as a single entity, no evidence of dissimilarity having been

found even between strains isolated at wide intervals in space and time, nor between "urban" and "jungle" strains (62).

Modification of yellow fever virus in tissue culture is best illustrated by the 17D strain, used for human immunization. Its development has been recently summarized by Sawyer *et al.* (67). This strain lost its viscerotropic virulence early, as tested by intraperitoneal injection of monkeys. Its ability to infect monkeys after intracerebral injection was retained for about 100 passages, and was then found to be reduced. Later passages were used for preparation of human vaccine. Numerous attempts have been made to induce this modification again with the Asibi and with other strains without success, as stated by Sawyer *et al.* (67) and by Theiler (62). The particular factors responsible for the modification of strain 17D remain obscure. Other types of variation in yellow fever virus are also reviewed by Sawyer *et al.* (67).

*Poliomyelitis.*—Although contributions to the subject of immunologic types among strains of poliomyelitis have been made over a period of many years, only recently have reports appeared of a systematic attack on the problem. Kessel & Pait (68) and Bodian, Morgan & Howe (69, 70, 71) have broken down a number of strains into three groups on the basis of several different immunologic tests. The two reports are apparently in agreement insofar as they are comparable. Bodian (69) has briefly reviewed the pertinent literature.

*Psittacosis group.*—The viruses comprising this group are closely related morphologically and immunologically, having a characteristic developmental cycle, and possessing an antigen in common as revealed by the complement-fixation test. Numerous reports on cross-immunization studies between various members of the group have revealed immunologic relations of varying degree. Wagner, Golub & Andrew (72) have reported recently on this subject. In contrast to the relationships revealed by complement fixation and cross-immunity tests, Hilleman (73) and St. John & Gordon (74) reported that cross-neutralization tests with antiserums prepared in roosters revealed immunologic specificity with almost all strains tested. The viruses of this group have been isolated from man (several strains) and a variety of animals. Isolations were made in various ways and strains have been cultured in mice and in eggs. One wonders whether some of the observed immunologic heterogeneity in this group may be due to antigenic variation after isolation.

*Other viruses.*—Available space permits only mention of certain other groups. Mengo encephalomyelitis, encephalomyocarditis, Columbia-SK, and MM viruses constitute a recently described group (75, 76). The same may be said for the Coxsackie viruses (77). The *Rickettsiaceae* represent a group, comparable to the true viruses, with many different naturally-occurring strains (78). The immunologic heterogeneity of strains of one species, *Rickettsia tsutsugamushi*, has been studied recently by several groups of investigators [cf. (79)]. The herpes group has been discussed from the evolutionary standpoint by Burnet (3). A neurotropic variant of dengue virus with potential value for human immunization, was recently described by Sabin & Schlesinger (80).

## Mechanisms of Variation in Viruses

*Mutation.*—It may be seen from the foregoing that the great majority of virus variants that have been observed to arise in the laboratory have appeared when a virus strain is placed upon a new medium, either a different animal species or a different tissue of the same species. The most reasonable explanation at present for this kind of variation is that growth in the new medium has resulted in the selection of a spontaneously occurring variant (mutant) by virtue of an advantage over the parent strain possessed by the variant in the new environment. Evidence for such a mechanism in bacteria has been repeatedly described (81, 82).

The apparently spontaneous change of the original strain of rabbit fibroma to one of quite different histopathologic character, as described by Andrewes & Shope (83), is probably explainable on a similar basis, but the possible involvement of an unknown mutagenic factor cannot be excluded.

Studies of certain variant strains of plant viruses indicate that their unit particles are of the same size (84, 85) and shape (85) as those of the parent strain. Stanley & Knight (86) found small physical and chemical differences among the purified preparations from a number of different strains of tobacco mosaic virus. Further investigations (87, 88) revealed differences in amino acid content of the proteins of such strains. Serological studies on the differences between plant virus strains have been pursued by numerous investigators over a period of years, and are continuing (89).

Gowen, in his summary (90) of observations on mutation in different organisms, reports data which he believes are indicative of mutation rates in tobacco mosaic virus. The frequency of mutation of the latter to aucuba (a mutant virus) was estimated to be $15.0 \times 10^{-4}$. The corresponding figure for the reverse mutation is $14.8 \times 10^{-4}$. These frequencies were determined by counts of the different types of lesions on susceptible leaves inoculated upon the surface with suitable dilutions of the virus. Gowen points out the difficulty of translating such figures directly to particle counts, because the observed rates obtained by this technique depend not only upon the original mutants but also the progeny of such mutants. The data are therefore obscured by unknown factors. Gowen (90) also reports observations on the mutagenic effect of x-rays on the virus. Opatowski (91) examined Gowen's data and calculated the volume within which the primary process of mutation presumably developed, based on the volume being occupied by protein, nucleic acid, or phosphorus. When the volume was calculated on the assumption that it consisted of protein, it was found to be identical with that of the hexagonal basic unit of the virus as described by Bernal & Fankuchen (92) in x-ray diffraction studies. Opatowski (91) also examined the relation between induced and spontaneous mutation rates and concluded that the latter could not possibly be due to the ionizing radiations which occur naturally (cosmic rays, $\gamma$-rays from radioactive minerals).

In work with animal viruses, two tentative estimations have been made of mutation rates: Burnet (3) estimated that one particle of D phase virus appears among $10^5$ to $10^6$ particles of the O phase. Ginsberg & Horsfall (93)

estimated that a polysaccharide-resistant variant of mumps virus (see below) was present among normal virus particles in a ratio of $1:10^4$ or lower. These rates are to be compared with estimated mutation rates of bacteria of $1:10^5$ to $1:10^{10}$ (94), of bacteriophage of approximately $10^8$ (95), and of tobacco mosaic virus as given above.

The absence of any satisfactory method of quantitation, especially in the case of animal viruses, makes it impossible to determine, with any degree of accuracy, either spontaneous or induced mutation rates. Without a reasonable estimate of spontaneous mutation rate, induced mutation cannot be detected.

It appears proper in this section to discuss the transformation, as it was originally called, of fibroma virus of rabbits into virus of infectious myxomatosis. These viruses, although producing different types of disease in rabbits, are closely related immunologically. Berry & Hedrick (96, 97) first reported that inoculation of fibroma and heat-inactivated myxoma viruses into a rabbit resulted in the appearance of typical myxomatosis. The analogy with transformation of rough pneumococci into smooth in the presence of type specific polysaccharide was pointed out. The observation has been repeatedly confirmed [cf. (98)]. The transforming principle has been shown to reside in the elementary bodies of the myxoma virus and is destroyed by heating to 90°C. but survives 60°C. and 75°C. Berry (97) interpreted the transformation as due to the transfer of a factor accounting for virulence from the antigenically more complex myxoma to the related fibroma. Both he and Gardner & Hyde (98) indicated an alternative interpretation, i.e., that the fibroma virus reactivates the inactive myxoma virus, inducing no change in character, but rendering it once more capable of multiplication.

Lindegren (99) has pointed out that the demonstration of a number of selective agents that have the effect of preventing the establishment of mutants of one type or another among bacteria [e.g., selective factor of Braun (100)] raises the question of whether such phenomena as the transformation of pneumococcus types may not also involve selection of spontaneously-occurring mutants. Applying such a postulation to the fibroma transformation, the transforming principle (heated myxoma virus) would be said to act by virtue of an inhibitory effect on fibroma virus, allowing a spontaneously-occurring mutant to grow. Under natural conditions, the mutant would always be overgrown by the parent strain. In this connection, Gardner & Hyde (98) indicated that the transformation appeared to take place more readily in older rabbits in which fibroma developed more slowly.

Appearance of a succession of antigenic variants in certain blood infections with *Borrelia* and *Trypanosoma* is well known [cf. (101)]. The recurring febrile episodes of equine infectious anemia led Watson (102) to suggest that antigenic variation may play a part in the course of that virus disease as well. Data such as these raise the question whether antiserum can act as a directive mutagen on disease agents infecting animals.

The possibility of such an effect is indicated by the studies of Sonneborn (103, 104) who reported that specific antisera against *Paramecium* are capa-

ble of inducing permanent alterations of antigenic characteristics, presumably by action upon plasmagenes. In earlier work, Harrison & Fowler (105), had noted effects of antiserum on *Paramecium* but attributed them to a selective action. Emerson (106) has discussed the possible direct effect of antiserum on genes. His postulations are worth consideration in respect to viruses also.

*Selective factors.*—The appearance of variation after introduction of virus into a new host species or a new tissue has many examples. But it must be apparent also that more subtle differences in environmental factors influence selection of mutants. It is stated [cf. (2)] that the experimental passage of street virus of rabies through a series of dogs by intracerebral injection may result in modified virus in the same manner as passage through rabbit brain. The selective factor here might be the bypassing of a whole series of tissues such as peripheral nerves, salivary gland, and muscle. It is also possible that variation in dosage may tend to favor or disfavor a spontaneously-occurring variant, as discussed above. The size of dose of O phase influenza virus injected in the amniotic cavity in Burnet's hands (36) determined whether variation to the D phase would occur.

Even more subtle factors in the host are its own genetic make-up, and its nutritional state. The influence of the latter on susceptibility to infection has been reviewed by Clark *et al.* (107). In regard to genetic factors, the work of Webster (108) is well known in which strains of mice were produced with low or high susceptibility to virus encephalitis. Many other instances of differences in susceptibility of strains of mice have been reported. Greiff & Pinkerton (109) found that embryonated eggs from different strains of chickens varied greatly in their ability to support growth of typhus rickettsiae. The influence of genetic character of the host upon susceptibility to various strains of plant viruses has been appreciated for some time [cf. (4)], and the genetic control of susceptibility of bacteria to bacteriophage mutants is well known (110). In the case of animal viruses few characters are available for study other than the nature of the disease produced. Since host factors are important in determination of the type or degree of infection produced, genetic definition and control of the host would seem necessary before genetics of the infecting virus can be thoroughly studied.

Arthropod-borne viruses deserve special attention with respect to the potentiality for variation associated with that kind of transmission. It has been pointed out by Huff & Coulston (111) that "mosquitoes infected with a particular species of *Plasmodium* must inoculate them promiscuously into hosts which present a wide variation in susceptibility." This must be true also of other microorganisms that are mosquito-borne, and further, it must occur with microorganisms that are transmitted by other arthropods with a wide host range, such as ticks and mites. It thus appears that arthropod transmission with such species would provide a constant multiple choice of medium for the microorganism. Mutants occurring spontaneously would tend to have a greater chance of finding favorable conditions for survival and growth, than in the case of transmission within a single host species. In

the interplay of potential transmission among multiple vertebrate and arthropod hosts, a large number of mutants might be expected to find an opportunity to establish themselves. Such a mechanism could account for the evolution of the many strains of arthropod-borne encephalitides, discussed above, and for the various strains, subgroups, and groups of rickettsiae [cf. (78)]. The mosquito-borne virus of yellow fever, however, appears to possess a remarkable degree of immunologic homogeneity, as mentioned above. This virus may be more stable genetically, or some other factors may be operating to limit the number of mutants that are able to establish themselves.

*Drug-resistance.*—By repeated passage of psittacosis virus in the presence of sulfadiazine, Golub (112) was able to produce a strain resistant to relatively high concentrations of that drug. Resistance was retained through a number of passages in the absence of the drug.

Unexplained variations in the results of quantitative experiments on inhibition of mumps virus by a polysaccharide, performed by Ginsberg, Goebel & Horsfall (113), led to the postulation that the particles in a preparation of mumps virus were not homogeneous with respect to susceptibility to the polysaccharide. Experiments (93) designed to separate out a polysaccharide-resistant variant were successful when passages of the virus were made in the presence of polysaccharide. Such variant strains retained their resistant character for a few passages in the absence of polysaccharide but eventually reverted to the previous polysaccharide-susceptible state. The two strains, susceptible and resistant, appeared to be identical in all other properties studied (immunologic character, infectivity, hemagglutinating properties) except one. A small difference was found in the rates of growth in the allantoic cavity of the egg, the susceptible strain possessing a slightly greater growth rate. This property is probably significant in reestablishment of the susceptible parent strain from the variant. Based on evidence regarding the mechanism of the polysaccharide inhibition, which is regarded as an effect upon the susceptible cell rather than upon the virus particle, the authors postulate that the resistant variant differs from the parent in biochemical requirements.

The development of resistant strains of viruses in the manner described in the preceding examples is best explained as the selective effect of the inhibiting substance in a population showing spontaneous mutation in the direction of drug resistance. Evidence for this kind of mechanism in the development of drug-resistant strains of bacteria is given by Demerec (114).

## Technical Limitations

No technique has yet been developed by which a single particle of a plant or animal virus can be selected out and cultured. Although inoculation of varying dilutions of a preparation can define within certain limits a single infective dose, few studies have attempted to relate infective dose to elementary body. [For exceptions see Parker & Rivers (115) and Parker (116)]. Thus, no investigations with these agents have been performed with progeny

of single particle isolates (clones). Until this is done, the question will persist, in any given experiment in which a variation has occurred, whether the observed result was due to selection from a mixture of two or more types present originally. In the case of some long-established strains, the possibility of a mixture seems remote but it cannot be entirely dismissed. Sugg & Magill (117) have demonstrated that two influenza strains, an A and B, can be successfully passaged together for a number of generations through mice and eggs. They point out that two or more strains with less profound differences might be expected to persist for a longer period in each other's company and thereby behave as a single strain. If such a "strain," of unrecognized dual make-up, were passed to a host tissue of such character that one component would overgrow the other, a variation in the "strain" would apparently occur.

Little fundamental advance can be expected until techniques allowing more refined quantitative observations are developed. It is not apparent whether these can be evolved from present techniques, such as the application of appropriate dilutions of plant viruses to the surface of leaves to produce discrete lesions, or the similar production with animal viruses of discrete "pocks" [cf. (118)] on the chorio-allantoic membrane of embryonated eggs. The need for constancy in the medium upon which viruses are grown, by control of genetic and nutritional factors in the host, has been mentioned above.

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# CURRENT TRENDS OF EXPERIMENTAL RESEARCH ON THE AQUATIC PHYCOMYCETES

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A recently published essay (28), reviewing a major field of biology, began with the refreshingly forthright statement that the account was "to be regarded as a short anthology selected entirely according to the author's prejudices." There is much to be said for a similar approach to research of the past ten or twelve years on the biology of the water fungi, for the entire spread of literature in this field is beyond our present scope, and the moment selections are made personal interests and beliefs immediately come into play. Hence it is well to understand that the following discussion is not intended to be an exhaustive review of papers, but rather an attempt to examine and integrate certain recent trends in the study of the aquatic Phycomycetes. The timeliness of such a survey is clearly indicated by a number of conditions which now exist. In the first place, there has been no review of advances in our overall knowledge of these fungi since Sparrow (69) published his very helpful analysis of the situation in 1935. In the second place, an important change in outlook is evident in many of the works currently dealing with the lower Phycomycetes. Increasing emphasis is being placed upon physiological aspects, many more investigations are being approached from an experimental point of view, and disciplines such as genetics and biochemistry are bringing new strength to a fabric which has been woven hitherto mainly of systematic and morphological studies. And, finally, concurrently with this broadened outlook, has come wide recognition of the fact that the aquatic fungi lend themselves very beautifully to the elucidation of certain biological problems of fundamental significance. The whole subject, then, has come into focus as an area of basic research in the life sciences. The erroneous and too widely held supposition that mycology is synonymous with taxonomy of the fungi is certainly not tenable now, if it ever was in the past.

No one in recent years has done more to provide us with a firm foundation upon which to build our experimental studies of the aquatic Phycomycetes than Sparrow. His monumental treatment of these organisms (71), published in 1943, though dealing primarily with their systematic position and relations, likewise contains a wealth of interesting material on many phases of their general biology, which serves as a priceless point of departure for the present review. To emphasize, however, the broad and fundamental nature of the problems involved, to show how they are related to each other, and to indicate the present state of our knowledge, the following discussion has deviated from the taxonomic viewpoint per se and has been organized around

the following major topics: (*a*) the use of pure cultures, (*b*) nutrition and metabolism, (*c*) germination of resting spores, (*d*) sexuality, (*e*) life cycles and nuclear cytology, (*f*) genetics and cytogenetics, and (*g*) experimental taxonomy.

### The Use of Pure Cultures

Considering that over half a century has elapsed since pure cultures became a commonplace in bacteriological research and that they are now almost universally recognized as an essential feature of microbiological investigations, one might suppose that the matter is undeserving of any special attention in the present discussion. Those who are familiar with the problem, however, will realize that an anomalous situation has existed in certain groups of the aquatic fungi until very recently.

It is true that pure cultures have played a significant role in investigations of most of the commonly occurring Saprolegniaceae and Pythiaceae for many years. These organisms, most of which are rapidly-growing, filamentous types, capable of development on a wide variety of natural and synthetic media, can be isolated readily and freed of bacterial contaminants by simple means which have been in general use since the early 1900's (46). It is usually quite sufficient to place a few washed, young hyphae on a dilute nutrient agar, such as peptone-dextrose, potato-dextrose, and corn meal. Within a few days the advancing tips of the rapidly spreading hyphae outgrow any contaminants and can be used to make transfers which will then be pure. Raper's (54) clever little device for limiting the spread of motile contaminants over the surface of the agar by surrounding the inoculum with a van Tieghem ring can be very helpful, but is rarely essential. The directness and simplicity of these methods probably account, in part, for the fact that investigators of the Saprolegniaceae and Pythiaceae have not ordinarily employed the slightly more painstaking but more precise procedure of obtaining pure cultures by making single spore isolations. As we shall see shortly, however, this technique, which is one of the basic features of research with most microorganisms, has been forced upon the attention of students of the water molds in recent years and is proving, as might have been foreseen, to be of wide applicability and importance.

Turning now to the other major groups of aquatic Phycomycetes we find a very different situation. Prior to 1940, only two of the 300 or more described species of Chytridiales, distributed in over 50 genera, had been isolated in pure, bacterium-free culture. In the Blastocladiales, *Blastocladia* had defied repeated attempts at isolation, and although the genus had been known since 1878 and the 12 or so species had often been collected in many parts of the world, no record of pure culture had appeared. Nor was there any account of the isolation of the classic genera *Monoblepharis* and *Gonapodya* in the Monoblepharidales. Of the six genera included in the Leptomitales, *Mindeniella* had not been cultured and *Rhipidium* and *Araiospora* had not been brought into pure culture since Minden's first report (51) of successful isolation in 1916.

Table I reveals the significant and very encouraging change in this picture which has taken place during the past ten years. The taxonomic groupings which are shown conform for the most part to those presented by Sparrow (71). The species of *Blastocladiella* are named in accordance with the revision of this genus by Couch & Whiffen (18), and *Catenaria* is included in the Blastocladiales where it has been placed by Couch (16). *Allomyces* has been omitted from the list of Blastocladiales, because all five species have been repeatedly isolated without any special difficulty (21). For the same reason *Leptomitus* and *Apodachlya* are not listed with the Leptomitales. It should be understood that, with the exception of the "two-membered" cultures of *Rozella* and *Woronina*, the term "pure culture" is used here in its usual strict sense to mean a culture containing only one kind of living organism. Some confusion has been created in the literature by the use of such expressions as "pure fungal culture" and "single spore culture" for isolates which were not bacterium-free and do not satisfy the accepted definition of a pure culture *sensu-stricto*.

We may ask, why is it that cultural studies have lagged so far behind the morphological and systematic investigations of these particular groups of aquatic Phycomycetes? And what are the points of particular significance which have been revealed by the work of the past decade on methods of isolation and purification? Because the writer is convinced that research with pure cultures is of the utmost importance if we are to gain a sound understanding of the aquatic fungi and if they are to play their rightful part in the elucidation of many fundamental biological problems, it will be worthwhile to analyze these questions briefly here and see what possibilities lie ahead. Several different factors need to be considered.

*Collection.*—Without question the unusual methods required to detect and collect these organisms in nature were originally responsible for the lack of first hand intimate knowledge about them exhibited by most biologists. The great majority of aquatic Phycomycetes rarely show themselves in sufficient quantity in nature to be gathered as one might harvest algae from a "bloom." Being either parasites or saprophytes, often with relatively specialized nutritional requirements which we are just beginning to understand, these fungi must be sought for on particular hosts or special substrata. The methods which have been employed are reviewed by Sparrow (71) and further details can be found in various earlier papers which he cites. In general, these methods involve the use of selected "baits" such as seeds, fruits, leaves, twigs, dead insects, algae, and so forth which are either added directly to water cultures in the laboratory or are set out in aquatic sites in nature. With increasing knowledge of the substrata and conditions required by the various groups of aquatic fungi, collecting has become more a science and less an art, and new techniques are being continually discovered. One of the most interesting developments in recent years has been the use of particular kinds of organic substances for the isolation of certain saprophytic species and especially members of the Chytridiales. In 1939 Haskins (29) and Couch (14) showed that pure cellulose is a good substratum for

TABLE I

RECORDS OF PURE CULTURES IN SEVEN ORDERS OF AQUATIC PHYCOMYCETES

| *Organisms* | *Year Reported* | *Isolation by* | *Citation* |
|---|---|---|---|
| Chytridiales | | | |
| Chytriomyces sp. | 1950 | Emerson | * |
| Cladochytrium crassum | 1941 | Hillegas | (32) |
| Cladochytrium hyalinum | 1941 | Berdan | (2) |
| Cladochytrium replicatum | 1939 | Couch | (14) |
| Cylindrochytrium Johnstonii | 1941 | Whiffen | (82) |
| Endochytrium operculatum | 1941 | Whiffen | (82) |
| Entophlyctis sp. | 1941 | Whiffen | (82) |
| Nephrochytrium aurantium | 1941 | Whiffen | (82) |
| Nowakowskiella elegans | 1941 | Whiffen | (82) |
| Polychytrium aggregatum | 1948 | Whiffen | (1) |
| Rhizophlyctis rosea | 1941 | Whiffen | (82) |
| Rhizophydium carpophilum | 1941 | Whiffen | (82) |
| Rhizophydium sp. | 1939 | Couch | (14) |
| Rozella Achlyae† | 1950 | Emerson | * |
| Rozella Allomycis† | 1950 | Emerson | * |
| Septochytrium variabile | 1941 | Whiffen | (82) |
| Blastocladiales | | | |
| Blastocladia Pringsheimii | 1940 | Emerson | (6) |
| Blastocladia ramosa | 1948 | Emerson | (22) |
| Blastocladiella simplex | 1948 | Emerson | (22) |
| Blastocladiella stomophilum | 1939 | Cox | (19) |
| Blastocladiella Stübenii | 1939 | Stüben | (76) |
| Blastocladiella variabilis | 1938 | Harder & Sörgel | (27) |
| Catenaria Anguillulae | 1945 | Couch | (16) |
| Monoblepharidales | | | |
| Gonapodya polymorpha | 1950 | Emerson | * |
| Gonapodya sp. | 1948 | Emerson | (22) |
| Monoblepharella elongata | 1947 | Stanier | (74) |
| Monoblepharella Laruei | 1947 | Stanier | (74) |
| Monoblepharella mexicana | 1950 | Stanier | * |
| Monoblepharella Taylori | 1943 | Sparrow | (71) |
| Monoblepharis polymorpha | 1950 | Emerson | * |
| Monoblepharis sphaerica | 1948 | Emerson | (22) |
| Anisochytridiales‡ | | | |
| Rhizidiomyces apophysatus | 1941 | Whiffen | (82) |
| Rhizidiomyces bivellatus | 1939 | Nabel | (52) |
| Plasmodiophorales | | | |
| Woronina polycystis† | 1939 | Couch | (14) |
| Lagenidiales | | | |
| Lagenidium giganteum | 1935 | Couch | (13) |
| Myzocytium sp. | 1939 | Couch | (14) |

TABLE I—*continued*

| *Organisms* | *Year Reported* | *Isolation by* | *Citation* |
|---|---|---|---|
| Leptomitales | | | |
| Araiospora spinosa | 1916 | Minden | (51) |
| Rhipidium americanum | 1950 | Emerson | * |
| Rhipidium interruptum | 1916 | Minden | (51) |
| Sapromyces androgynus | 1950 | Emerson | * |
| Sapromyces elongatus | 1940 | Bishop | (4) |

* These isolations are reported here for the first time and further details about them will be given elsewhere at a later date. I am indebted to Dr. R. Y. Stanier for his kind permission to record the isolate of *Monoblepharella mexicana*.

† These parasites were grown on their hosts in two-membered, bacterium-free culture.

‡ The new order Anisochytridiales was proposed by Karling (35) in 1943 to include chytrid-like organisms with anteriorly uniflagellate zoospores.

certain chytrids and that these forms can be isolated in cultures baited with pieces of filter paper or cellophane. Karling (36, 38, 39) reported a few years thereafter that purified chitin, from the exoskeletons of various Crustacea, supported a good growth of "chitinophilic" chytrids as he called them while keratin-decomposers developed well in cultures baited with skin, nail-pairings, hair, wool or feathers. Just recently Huneycutt (33) has described a new member of the Saprolegniaceae obtained in cultures provided with keratinized materials as bait. Emerson & Cantino (22) pointed out that the limitation of *Blastocladia* to certain natural substrata (apples, tomatoes, etc.) "provides the investigator with a method of collecting much like a sort of enrichment technique." Obviously, the use of fairly pure cellulose, chitin, keratin, or other organic substances comes much closer to representing a true enrichment culture in the bacteriological sense. Indeed, Stanier (73) described the mineral agar plate covered with a round of filter paper, which he used to isolate *Rhizophlyctis rosea*, as a "highly selective enrichment medium."

In summarizing this brief treatment of collecting methods, it should be borne in mind that a rich literature already exists on the subject, and fuller cognizance of it than has been evidenced by general microbiologists in the past is necessary if we are to make rapid progress in cultural research on the aquatic Phycomycetes.

*Restricted growth.*—Another factor which has been responsible for many past failures to obtain pure cultures of the groups of fungi listed in Table I is the restricted growth which characterizes many of them. Unlike the common molds, which develop an extensive mycelium capable of almost indefinite spreading as long as suitable conditions are maintained, the monocentric chytrids, *Blastocladiella*, and similar types, form only a single reproductive center and a limited vegetative system. Even in those forms like

*Blastocladia* or *Rhipidium* which may have a branched, semifilamentous structure, growth of an individual plant is definitely limited in extent. For this reason, the simple methods of purification so widely applicable to filamentous forms (see above) can hardly ever be employed, and even attempts to use them with the slowly spreading polycentric chyrids have quite consistently led to failure, with the exception of Berdan's (2) isolation of *Cladochytrium hyalinum* in 1941. Clearly, then, the inapplicability of the techniques generally applied to filamentous fungi was responsible in some measure for the delayed isolation of the organisms we are considering.

*Pure cultures from zoospores.*—Although the surrounding water gives bacteria and other contaminants immediate access to all the external surfaces of aquatic fungi, their motile spores are borne within closed sporangia. Almost invariably, furthermore, the release of these zoospores can be brought about at will when mature plants are collected from nature or taken from gross cultures and placed in clean fresh water. After their emergence from sporangia, the spores usually swim freely for some time. Here, then, would appear to be ideal material for obtaining pure, single-spore cultures directly by ordinary bacteriological techniques. Yet the literature, even in recent years, reveals relatively few instances of pure culture of aquatic Phycomycetes by these simple, well known means. One is forced to the conclusion that most of the very men who became so adept at collecting these fungi, were, in turn, either unaware of bacteriological methods or disinclined to try them out. Minden (51) was certainly an exception in this regard, and it is interesting to read his lucid description of the simple way he isolated freshly discharged zoospores in little drops of water and achieved the first pure cultures of *Rhipidium* and *Araiospora* as early as 1916. Significantly enough the latter genus has not been isolated in pure culture by anyone since then, and the former was reisolated only last year by the writer.

For nearly 20 years following Minden's pioneering work, few mycologists appear to have devoted much serious attention to the problem of obtaining pure cultures of the groups of fungi listed in Table I. In the 1930's, several separate studies were finally undertaken, and we are indebted to Couch (14) for the first significant publication on pure cultures of the Chytridiales. Couch's methods of plating zoospores were soon used by others [Cox (19); Hillegas (32)] and with some modifications and outstanding success by Whiffen (82) who reported pure culture of nine species of chytrids in 1941. In 1942, Stanier (73) made a particularly important contribution by his critical analysis of the pure-culture problem and his application of simpler, more straightforward microbiological techniques to the isolation of *Rhizophlyctis rosea.* He bypassed the initial laborious process of isolating single sporangia, which had been so much emphasized by Couch, and showed that pure, single-spore cultures could readily be obtained simply by streaking zoospore suspensions on a suitable agar medium. His success in this was aided by the acute observation that the spores of *Rhizophlyctis*, being strongly aerotactic, accumulated in large numbers at the surface of the gross cultures, thus providing inocula for streaking with a minimum of contaminants.

In 1948, Emerson & Cantino (22) published a detailed account of the methods which they used to obtain the first pure cultures of *Blastocladia*. They showed that zoospores can be neatly and rapidly collected in the capillary stream of a sterile micropipette, without the complication of an attached syringe, and described how zoospores were transferred in this way and washed through several dishes of sterile water in order to eliminate bacteria and other microorganisms which are so particularly abundant in the dense pustules of *Blastocladia* occurring on natural substrata. Similar methods were used to isolate *Monoblepharis sphaerica* and *Gonapodya* sp.

In the last two years, by combining the techniques applied to *Blastocladia* with the streak-method so successfully employed by Stanier, the writer has with very little difficulty made several more additions to the growing list of pure cultures (cf. Table I, 1950 entries). For streaking the zoospores, which appear to be rather more delicate in some of these forms than they are in such genera as *Saprolegnia*, *Pythium* or *Allomyces*, a small curved glass rod was used. It is also worth noting that, although zoospores of all other species were released in goodly numbers at ordinary laboratory temperatures, those of *Rhipidium americanum* would not emerge from the sporangia unless the cultures were cooled to about 15°C. This may well be one reason why later investigators had difficulty in repeating Minden's isolation of *Rhipidium*, and it is a point to be borne in mind in future work since this entire technique of obtaining pure cultures hinges upon abundant release of zoospores.

*Culture media.*—Admittedly, research on all phases of the nutrition and metabolism of living organisms has made tremendous strides in recent decades. Yet here, too, the evidence indicates that most past students of the water fungi had either too little interest or too scant knowledge to apply certain basic principles of nutrition and growth to the problem of obtaining pure cultures. Even in the more recent literature some rather anomalous statements appear. Knowing, for instance, that all organisms require an available supply of nitrogen as well as carbon for growth, it is not surprising that certain chytrids which developed on cellulose in gross cultures were unable to grow on boiled filter paper in water (14) when no source of nitrogen was provided.

At least four important points ought to be borne in mind in making initial isolations of water fungi. The organism may tolerate only a rather narrow range of hydrogen ion concentration; aside from mineral constituents, it will require a supply of nutrients including a source of nitrogen, a source of carbon, and very possibly a source of growth factors; its zoospores may easily be injured or killed if the total osmotic concentration of the medium is high; and, finally, it may itself produce metabolic products which accumulate and rapidly become toxic, thus inhibiting growth and necessitating early transfer.

Although not generally recognized, a sizeable amount of valuable information is already available on several of these points. Whiffen (82) successfully employed media for the chytrids with pH adjusted to 7.2, and Emerson & Cantino (22) reported that *Allomyces* and *Blastocladiella* will grow well between pH 6 and 8. *Blastocladia* on the other hand (22) does very poorly

in neutral media and shows optimum growth at about pH 6.5. The writer has found recently that *Rhipidium* responds to pH much as does *Blastocladia*, while *Sapromyces* is similar to *Allomyces* in this regard.

In those instances where no specific information is available regarding the nutritional requirements of a fungus, the various natural media, which have so long been used by mycologists, are still extremely useful, since they can be expected to supply adequate amounts of all the organic nutrients which might ordinarily be required. Such standaradized products as the corn meal agar and prune agar of the Difco Laboratories give transparent preparations that permit detailed microscopic observation of zoospores and germlings. Semisynthetic media, provided with yeast extract, beef extract, or peptone, and some generally available carbohydrate like glucose, also serve well. The writer's recent isolations (cf. Table I) of *Rhipidium*, *Gonapodya* and *Sapromyces* were made with glucose-yeast media similar to medium GY3 of Emerson & Cantino (22); the species of *Monoblepharis* were isolated on agar containing ½ per cent tryptone which Stanier (personal communication) had found supported good growth of *Monoblepharella*. When gross enrichment cultures, like those previously mentioned, have given definite indication of some of the nutritional characteristics of an organism, more selective media can often be used to advantage. Thus, Stanier (73) and Whiffen (82) both employed mineral-glucose or mineral-cellulose media in isolating cellulolytic chytrids. The information which Karling (36, 38, 39) and others have already obtained regarding the chitin- and keratin-decomposing chytrids should prove very useful in future efforts to isolate these forms in pure culture.

Some of the water fungi, as might be expected, seem unable to tolerate high osmotic concentrations, and media containing relatively small amounts of salts and nutrients are certainly advisable for initial isolations. Dilute media also induce more rapid spreading of the filamentous forms, often allow normal zoospore emergence, and tend to reduce the rate of multiplication of bacterial contaminants.

A striking illustration of the importance of watching for toxic metabolic products was revealed in the work of Emerson & Cantino (22) on *Blastocladia*. This organism produces acidic products so rapidly from glucose that the pH of the medium close to the colonies drops quickly below the lower limit of tolerance, and transfers have to be made every few days in order to maintain viable material on agar. That these observations are of more than isolated significance became clear recently when the writer found that *Rhipidium* and *Sapromyces* are also strong acid producers which require frequent transfer when they are cultured on agar media. Cantino (9) has just reported that *Pythiogeton* too forms acids when carbohydrates are metabolized.

As has already been indicated, many of the aquatic Phycomycetes are parasitic in nature on a wide variety of plant and animal hosts. While some are clearly facultative and quite capable of saprophytic growth, others appear to be wholly dependent upon their host(s), and of these the holo-

carpic forms in the Chytridiales, Lagenidiales, and Plasmodiophorales in particular present many baffling problems. To date none of these organisms has been isolated in pure culture, but the possibility of making controlled studies of their biology has been greatly enhanced by successful efforts to grow three of the species with their hosts in pure, two-membered cultures. In 1939, Couch (14) listed such a culture of *Woronina polycystis* growing parasitically on its host, *Achlya flagellata*, and free of all contaminants. No further details were given, but it is to be hoped that further developments will be forthcoming from this very significant achievement. Similar two-membered cultures of *Rozella Allomycis* on *Allomyces arbuscula*[1] and *Rozella Achlyae* on *Achlya* sp. have also been isolated on nutrient agar by the writer, and the *Rozella Allomycis* is presently being used in our laboratories for a study of host-parasite relations.

## Nutrition and Metabolism

Significant advances have been made during the past ten years in our knowledge of the nutritional requirements of certain groups of the aquatic Phycomycetes. In part, this is a result of the widespread interest which exists in the nutrition of animals and plants at all levels of specialization, but it is also more specifically a direct outgrowth of recently successful attempts to obtain pure cultures of species of water fungi never previously isolated (cf. Table I). Nutrition is necessarily related directly or indirectly to all life processes and as such is a subject well worthy of attention in the present discussion. Since, however, Cantino (10) has just published a concise and comprehensive account covering virtually all of the work done to date on the synthetic capacities of the water molds, the matter will not be dealt with here.

The information presently available on the metabolism of the aquatic Phycomycetes is, unfortunately, still very limited. Only two genera have been investigated intensively: *Leptomitus* by Schade & Thimann (64) in 1940 and *Blastocladia* by Emerson & Cantino (22) in 1948 and Cantino (8) in 1949. Limitations of space prohibit discussion of the interesting results reported in these papers, but the reader is referred to critical comments about them which appear in Foster's new book (25) on the chemical activities of the fungi and lend emphasis to the great need for further work along these lines.

## Germination of Resting Spores

Aside from simple walled off portions of hyphae, like the gemmae of the Saprolegniaceae, the true resting spores of the aquatic Phycomycetes, often also referred to as resting or resistant sporangia, are of two major

[1] I wish to correct here an error which I perpetrated in 1941 [(21) ff. p. 78]. At that time I believed that the proper spelling was *arbusculus* rather than *arbuscula*. Torrey (78) has pointed out the mistaken nature of my argument. I profoundly regret the very unnecessary confusion which I created.

sorts, those that develop directly from a zygote and those that do not, that is to say those that are sexually produced and those that are asexually produced. The former type, which is much the more usual, is exemplified by the oospores of the Peronosporales, Leptomitales, Saprolegniales, and Monoblepharidales, as well as the resting spores of the Lagenidiales and certain members of the Chytridiales. Asexually produced resistant spores occur in all genera of the Blastocladiales and in some Chytridiales. For many reasons, the resting structures are frequently of critical importance in biological studies of the aquatic Phycomycetes, yet we are forced to admit that we have as yet only the most meager knowledge regarding the physiology of their formation or germination. Many taxonomic descriptions and identifications remain inadequate because resistant spores could not be found or germinated. Life-cycle studies have been blocked, investigations of the nuclear cytology of germinating oospores have been thwarted, and genetic analyses of hybrids have often been prevented. Most of the research on resting spores has been of the trial-and-error variety, and too frequently the results reported are difficult to interpret or repeat owing to the use of impure cultures and poorly controlled conditions. A few specific contributions made in recent years deserve emphasis.

Considering first those groups in which resting spores are sexually formed, an advance of a very practical nature was made by Ziegler (88) in 1948 when he succeeded in germinating the oospores of some 26 species of Saprolegniaceae, a family notorious for the many unsuccessful attempts which had been made previously to germinate the spores. Apparently, the most important single factor is the time required for maturation, which varied from about 3 to 24 weeks depending upon the species concerned. Under the conditions which Ziegler employed, where the fungi were grown in pure water-culture on hemp seeds, it was shown that when oospores were fully mature the only stimulus required, with few exceptions, was the change of conditions involved in transfer from the old culture to fresh water. Temperatures above 30°C. and pH values which deviated by more than half a unit from neutrality were unfavorable for oospore germination. Unfortunately, neither the conditions of maturation nor those for germination were fully controlled, but it seems evident that a thorough study of the various factors involved in maturation would be most worthwhile and could very possibly reveal ways to shorten the maturation period. Blackwell (7) has devoted much attention to the problem of germinating the oospores of *Phytophthora* and in 1943 described and discussed the complex experiments which she and her students had done during the previous 15 years. Her conclusions indicate that the spores of *P. cactorum* can be induced to germinate by a rather elaborate treatment involving a period of maturation for 2 to 8 months at 10°C. to 15°C. under moist conditions, followed by one or two weeks at about 1°C. and subsequent transfer to alkaline water containing calcium ions. It is not clear whether pure cultures were used in this work. Shanor (65) likewise found in 1939 that a period of maturation extending to two or three months was required in

order to germinate the resting spores of *Olpidiopsis varians* which had developed as a parasite in impure water cultures of *Achlya flagellata*.

Turning to the Blastocladiales, we find again that maturation appears to be of fundamental importance. Despite many previous attempts, no one had succeeded in germinating the resistant sporangia of *Blastocladia* until 1937 when Blackwell (5) showed that mass germination took place in fresh water within 24 hr. if resistant sporangia had been previously imbedded in moist agar for two or three months. Controlled studies on the behavior of resistant sporangia of *Blastocladia* were not feasible until Emerson & Cantino (22) reported in 1948 that a high concentration of carbon dioxide is required to induce the formation of these structures in pure cultures, and Cantino (8) showed further that pH is also a critical factor. The conditions requisite for germinating resistant sporangia from pure cultures remain to be established. On the other hand, resting structures are usually formed in great abundance in pure cultures of *Allomyces* and *Blastocladiella* without special treatment. There are numerous references in the literature to indicate that desiccation is often a factor of importance in the maturation of the resistant sporangia of these genera, but Emerson (21) has pointed out that desiccation is not essential since resistant sporangia formed and held continuously in water cultures will sometimes germinate. It has also been shown (20, 24, 45) that resistant sporangia of *Allomyces* become capable of germination three to six weeks after their formation on agar slants. Emerson and Wilson (24) have demonstrated, moreover, that the mature resistant sporangia of *Allomyces* contain diploid nuclei in an advanced prophase of meiosis I and that during the process of germination, which takes 100 to 130 minutes at 20° to 25°C., the reductional divisions occur on a very precise time schedule. This discovery of meiosis in the asexually-formed resting structures of the Blastocladiales reveals a most interesting point of basic uniformity in many, if not all, aquatic Phycomycetes having sexual reproduction: whether sexually or asexually formed, the true resting spore appears to be the site of meiosis. We have seen that maturation is a factor of prime importance in germination, and very probably the changes which occur in maturation are intimately bound up with processes related to meiosis. Indeed, the resting spores of the water fungi may ultimately provide exceptionally favorable material with which to investigate certain aspects of the physiology of reductional chromosome divisions. In this connection, it is interesting to note that Hatch & Jones (31) found evidence which suggested that mitotic, rather than the normal meiotic, divisions may occur in certain strains of *Euallomyces* when the resistant sporangia undergo a short period of maturation (cf. LIFE CYCLES AND NUCLEAR CYTOLOGY).

Another significant aspect of germination was brought out in 1939 by Stüben's (76) studies of *Blastocladiella Stübenii* (= *Sphaerocladia variabilis*). When this organism was grown on media with a good supply of nutrient it formed large, thick-walled resistant sporangia which would not germinate despite a variety of special treatments. On a very dilute medium, however,

containing only 0.002 % peptone, the plants were severely starved and produced small resistant structures, with much thinner walls, which germinated easily when transferred to water. It may well be, as Stüben supposed, that germination was a result of the more ready penetration of water and rupturing of the thinner wall, but it also appears likely that starvation brought about more rapid maturation of the protoplasmic content of the sporangia, and the possible effects of nutrition on the maturation processes could well be investigated in other water fungi. Recent unpublished work of Lewin [referred to by Starr (75)] on the resting zygotes in *Chlamydomonas* has shown that here too starvation causes a thinner wall and more ready germination.

## SEXUALITY

Like the green algae, the lower Phycomycetes display a wonderfully diverse array of morphological and physiological adaptations for sexual reproduction. Compared with the basic uniformity of sexual processes shown in each of the major groups of higher fungi and algae, not to mention all higher plants, the primitive thallophytes appear to represent a testing ground in the evolution of devices associated with gametic fusion. Students of sexuality have found these plants ideally suited for experimental research, and general concepts of relative sexuality and the origin and evolution of sex have been built up against a background of such investigations. Kniep's classic volume (43) provides us with an exhaustive and analytical survey of work on the aquatic Phycomycetes prior to 1928. Thereafter, Sparrow (69, 71) effectively reviewed the various advances made in this field, first to 1935 and then to 1943. The present discussion will be limited to certain aspects which have seemed to the writer to be of more than ordinary interest and general significance.

*Chytridiales.*—If sexual processes are diverse in the aquatic fungi as a whole, they appear to show the maximum of diversity in the most primitive of the true fungi, the uniflagellate chytridiaceous types and their counterparts in the biflagellate series. Two basic types of sexuality, which appear to foreshadow clearly the sexual processes of the higher Phycomycetes, have been recognized, however. One is the fusion of equal (isogamous) motile cells, probably frequently so undifferentiated that they behave as asexual zoospores or sexual gametes, depending upon environmental conditions. The other is direct fusion of whole thalli or portions of thalli which, again, are often influenced in their sexual expression by external factors of the environment. Suggestions regarding possible instances of genotypic sex determination have been made by various investigators and schemes, such as that recently proposed by Karling (37, Diagram 1) for *Siphonaria* have been drawn up, but, to the writer's knowledge, there is not yet a single well-established example in the chytrids of a differentiation of sexes clearly attributable to segregation of genetic factors at the time of meiosis in the life cycle. In view of the wide-spread and very general occurrence of hermaphroditism and phenotypic sex determination in the higher aquatic fungi, and the very few

instances in which a genetic difference between the sexes is indicated, perhaps we should not expect to find more than scattered examples of genotypic sex determination in the lower forms. It is unfortunate in this regard that no one appears, as yet, to have verified or amplified Couch's (15) interesting experiments in 1939 which gave strong indication of heterothallism or haplodioecism in *Pringsheimiella* and *Rhizophlyctis*. With the techniques now available for single-spore and pure-culture studies of the chytrids, this should be a productive field of investigation.

*Isogamy, anisogamy, and sex determination in the Blastocladiales and Monoblepharidales.*—Kniep's (44, 45) discovery of motile anisogametes and an isomorphic alternation of generations in *Allomyces javanicus* was of immense significance, not only because it was the first demonstration of this type of sexuality and life cycle in the fungi, but also, and even more important, because it was the starting point of a whole series of noteworthy investigations on reproductive processes and heredity in the Phycomycetes. A full account of much of this work, with figures and descriptions of the organisms involved, will be found in Sparrow's (71) treatise. We must confine ourselves here to a brief examination of the main findings.

The anisogamous species of *Allomyces*, which constitute the sub-genus *Euallomyces* (cf. LIFE CYCLES AND NUCLEAR CYTOLOGY) provide exceptionally favorable material for experimental studies of phenotypic sex determination. The hermaphroditic sexual plants bear nearly colorless female gametangia and bright orange male gametangia directly adjacent to each other in terminal pairs. Each sexual thallus develops from a uninucleate, haploid spore and all nuclear divisions are mitotic so that the processes leading to the differentiation of the gametangia and gametes are directed by a single genotype. We have, as yet, little real evidence of the cytoplasmic changes involved, but the prospect of explaining this sex determination in concrete terms of differential biochemical processes is an intriguing one. Hatch (30) made the interesting observation that there was a differential distribution of mitochondria in the hyphal tips prior to the delimitation of the gametangia by cross walls. In *A. arbuscula*, the species he was studying, the female gametangium is terminal and the male is subterminal or hypogynous. Since the mitochondria were concentrated in the most apical portion of the hypha, the cell which was destined to become female received the major share of these cytoplasmic constituents. Hatch concluded that "the role of the chondriosomes is certainly of primary significance in the expression of sexuality." No statement has been made about mitochondrial distribution in *A. javanicus*, a species in which the male gametangium is epigynous, but if an excess of mitochondria is indeed related to, or causally connected with, femaleness, the mitochondria should congregate in a subapical portion of the hypha in this form. Emerson & Fox (23) extracted the orange pigment from the male gametangia of several different isolates of *Euallomyces* and found it to be nearly pure $\gamma$-carotene. They discussed the likelihood that this pigment is intimately involved in the sexual behaviour of *Allomyces*, but no data are

yet available to show its precise function or why it is synthesized only in the male cells.

Some light has been shed on the inheritance of the sex-determining mechanism in *Allomyces* by the interspecific crosses (see GENETICS AND CYTOGENETICS) which Emerson (21) made between *A. arbuscula* and *A. javanicus*. Many of the $F_1$ gametophytes were intermediate, bearing varying proportions of hypogynous and epigynous pairs of gametangia; hence it is clear that the sex-determining processes are under the control of two or more genes on different chromosomes of the haploid set. It is also interesting to note that the ratio of male to female gametangia was markedly changed in some of the hybrids. Male and female cells occur in approximately equal numbers in the parental strains, but gametangia of one sex often predominated in the hybrids, with ratios as high as 99 male to 1 female or vice versa in certain instances. In these forms many paired males or paired females occurred.

Kniep (45) and others (21, 68) have shown that the female gametes of *Euallomyces* are capable of developing parthenogenetically into perfectly normal gametophytic plants which bear both male and female gametangia. These experiments vividly demonstrate the bisexual potency of the haploid nucleii in *Allomyces* and provide one of the most striking examples of the inherent maleness of a markedly differentiated, uninucleate female gamete.

Weston (80) had suggested in 1935 that aquatic fungi with a less complex plant body and more primitive type of sexuality than those of *Euallomyces* would probably soon be discovered. This prophesy was fulfilled to an amazing degree within three years, when isogamous sexual reproduction was found in *Blastocladiella*, a very closely related genus in which the thallus is limited in size and ordinarily bears but a single reproductive structure. Harder & Sörgel (27) described in 1938 the two types of gametophytic plants which they had discovered in *B. variabilis*. One bears an orange-colored gametangium; the other a colorless one. They termed these plants "plus" and "minus" since the gametes from each are equal in size and fuse in pairs, but by analogy with the species of *Euallomyces*, which have an identical life cycle (see pp. 187–91), the orange gametangium can, in all probability, be considered "male" and the colorless one "female." Harder & Sörgel made the comment that sex determination in *B. variabilis* is probably genotypic, but presented no evidence to support this contention. Discovery of meiosis in the resistant sporangia of *Euallomyces* now makes it almost certain that the sexual plants of *B. variabilis* do develop from spores formed by meiotic divisions. Despite this, however, the writer has felt it unlikely that sex determination would be genotypic in *Blastocladiella* when it was so obviously phenotypic in *Euallomyces*. Experiments performed a year ago with a strain of *Blastocladiella*,[2] which is like *B. variabilis* in all essential respects, provided a definite answer to this question. Female gametes from a single gametan-

[2] I am much indebted to Dr. E. C. Cantino for the subculture of this organism which was isolated in his laboratory at the University of Pennsylvania.

gium were germinated parthenogenetically and developed into mature gametophytes. Plants of both sexes were unmistakably present in the population, the males each bearing an orange gametangium, the females each bearing a colorless one. Male plants continued to appear regularly thereafter in each of nine, consecutive, parthenogenetically produced generations, a fact which demonstrates clearly that sex determination in this *Blastocladiella* is phenotypic, just as it is in *Euallomyces*. There is no segregation of male and female determiners at meiosis; each haploid nucleus carries full potentialities for maleness and femaleness. It would seem that the sexual plants of *B. variabilis* are inherently just as hermaphroditic as those of *Euallomyces*. In *Blastocladiella*, however, each sexual thallus bears only one reproductive organ which must, perforce, be either male or female. There is, therefore, no justification for including *B. variabilis*, as Whitehouse (86) has done in his recent interesting review of heterothallism and sexuality, among those fungi which have sexual reproduction involving two genetically different thalli.

A still more primitive type of sexuality was added to the Blastocladiales in 1939 by Stüben's (76) discovery of strict isogamy in *Blastocladiella Stübenii* in which there is no pigmentary difference between the plus and minus gametangia. Stüben again concluded, from the approximately equal ratios of plus to minus thalli which he observed, that sexuality is genotypically determined at meiosis. It would be interesting to apply the test of parthenogenesis to this species also, but there may be greater difficulties, since Stüben reported that apomixis did not occur under the conditions he employed.

A contribution of fundamental importance was made when McCranie (49) and Teter (77) demonstrated that strictly isogamous sexuality also occurs in *Allomyces*, namely in *A. cystogenus* and *A. moniliformis*. In his studies of these species, which are included in the subgenus *Cystogenes*, Emerson (20, 21) had observed the encystment of zoospores from resistant sporangia (R. S. zoospores) and the subsequent release of swarmers from the cysts, but he had mistakenly concluded that the swarmers from cysts are asexual and therefore failed to recognize the sexual step in this cycle. From McCranie's preliminary account in 1942 and Teter's much more careful study which followed it became clear that the cysts are gametangia and that the uniflagellate swarmers which emerge from them are isogametes that fuse in pairs to form motile zygotes. Between 1942 and 1946 Couch & Whiffen (16, 18, 85) significantly extended our understanding of this type of sexual reproduction by their discovery and detailed observation of isogametes derived from encysted R. S. zoospores in *Blastocladiella cystogena*, *B. microcystogena* and *Catenaria Allomycis*. Thus, within four years of McCranie's first account, three genera and five species of the *Blastocladiales* were found to have isogamous sexuality associated with encystment of the zoospores from resistant sporangia. While there is general agreement among all investigators as to the striking uniformity of the main features of this process, certain points of difference will require further study. Present reports indicate that R. S. zoospores of the cyst-forming species of *Blastocladiella* and *Catenaria* are uniflagellate (16, 18, 85) and uninucleate (16), whereas those of

*Allomyces cystogenus* are usually biflagellate (20, 21) and binucleate (C. M. Wilson, personal communication). Since there appear ordinarily to be four gametes per cyst in all three genera, we must conclude for the present that two nuclear divisions are involved in gametogenesis in the cyst-forming species of *Blastocladiella* and *Catenaria* but only one in those of *Allomyces*. Some investigators (18, 77) have suggested that gametes from the same cyst will not fuse with each other, *i.e.*, that the cysts are separate sexed, but the isolations and mating tests required to substantiate this contention have not been reported. Apomictic development may well complicate the problem seriously. At any rate, when the necessary cytological and experimental evidence is at hand, it will be most interesting to find whether the cyst-forming Blastocladiales have genotypic sex determination or whether, as in most if not all of their long-cycle relatives, sex is established phenotypically.

Sexuality has not yet been found in *Blastocladia*, the type genus of the Blastocladiaceae, but certain investigations of the past 12 years have brought the problem much closer to a solution. In 1940, Blackwell (6) succeeded in observing for the first time a complete life cycle in *B. Pringsheimii*, and although she discovered no gametic phase in this particular isolate, her success in germinating the resistant sporangia (5) was obviously a fundamental first step in the search for sexuality in the genus. Emerson & Cantino (22) fitted another significant piece into the puzzle in 1948 when they showed that plants of *B. Pringsheimii* could be induced to form resistant sporangia in pure cultures if they were subjected to high concentrations of carbon dioxide. A systematic series of pure culture studies of all the twelve or so known species of *Blastocladia* should be undertaken to determine which, if any of them, do have sexual reproduction.

Great interest in the Monoblepharidales was reawakened by Sparrow's (70) discovery of *Monoblepharella* in 1940. This remarkable organism has large non-motile eggs and active uniflagellate antherozoids much like those described 70 years previously by Cornu in *Monoblepharis*, but following plasmogamy the zygote, instead of remaining within or attached to the mouth of the oogonium, becomes detached from the parent plant and swims away propelled by the protruding flagellum of the male gamete. There are several basic points of similarity between the sexual mechanisms of the Monoblepharidales and those of *Euallomyces* in the Blastocladiales. The sexual plants of *Monoblepharis* and *Monoblepharella* are believed to be haploid, they are self-fertile hermaphrodites, and they bear male and female gametangia in terminal pairs. Moreover, there are epigynous and hypogynous species in each genus just as there are in *Euallomyces*. Studies to show how the sex determining mechanism is inherited in interspecific hybrids would be most interesting and should be feasible now that epigynous and hypogynous members of both genera have been isolated in pure culture. However, the precise conditions requisite for sexual reproduction in pure culture remain to be established. Both Sparrow (71) and Springer (72) have stated that a change from asexual to sexual reproduction can be brought about in gross cultures by a rise of a few degrees in temperature. This effect

of temperature, however, appears to depend upon the complex conditions existing in gross cultures, since the writer has found that comparable changes in temperature are ineffective in inducing the formation of gametangia in pure cultures.

*"Heterothallism" and sexuality in the biflagellate series.*—One of the particularly interesting aspects of sexual reproduction in the aquatic Phycomycetes, which has received special attention in recent years, is the condition of so-called heterothallism occurring in certain genera of the Saprolegniaceae and Leptomitaceae. In these families by far the majority of species that reproduce sexually bear oogonia and antheridia on the same thallus; that is to say they are self-fertile hermaphrodites and can be spoken of as haplomonoecious or homothallic. Sex determination is phenotypic and examples of sex reversal are not uncommon. There are, on the other hand, a few species which have been found to exhibit a kind of dioecism or heterothallism involving the separation of the sexes in different thalli. Ideally, this involves the formation of antheridia by one mycelium and oogonia by the other. In actuality the situation is evidently considerably more complex than this and has not yet been fully clarified. The various investigations and their possible interpretations have been subjected to critical analysis by Raper (57) and discussed by Sparrow (71) in his book. Outstanding among these contributions are those of Couch (12) on *Dictyuchus monosporus* in 1926, Raper (53, 59) on *Achlya bisexualis* in 1936, and *A. ambisexualis* in 1940, and Bishop (4) on *Sapromyces elongatus* in 1940. From the wealth of experimental data obtained by these investigators with single spore strains grown in pure culture and mated in all possible combinations, an extremely significant and essentially consistent picture has emerged. There are (i) pure male and (ii) pure female strains which remain sterile when grown separately but produce antheridia and oogonia, respectively, when they are brought together. In addition, however, four other types of plants have been found: (iii) typical self-fertile hermaphrodites, (iv) neutral strains that have never formed antheridia or oogonia in any matings, (v) females with a latent capacity for maleness which can be demonstrated by matings with pure females, and (vi) males with a latent capacity for femaleness which can be demonstrated by matings with pure males. This kind of sexuality is evidently quite different from heterothallism as first defined in the Mucorales, and Raper has applied to it the term *gynandromixis* which implies that there is only partial separation of the sexes, three of the six possible sexual types showing various degrees of combined maleness and femaleness. Both Bishop (4) and Raper (57) have postulated that the sexual type of any given strain is determined by the presence of two out of four hypothetical factors in its haploid nuclei. These factors are defined by Raper (57, p. 720) as follows: F, dominant femaleness; f, latent femaleness; m, latent maleness; and M, dominant maleness. The six sexual types listed just above are considered to have the following genetic constitution: (i) Mm, (ii) Ff, (iii) FM, (iv) fm, (v) Fm, and (vi) fM. If we suppose that F and f are alleles of M and m respectively, and that there is no linkage, a cross between Mm (pure male) and Ff

(pure female) would give MFmf zygotes, and random segregation at meiosis would give types (i), (ii), (v), and (vi), but some special mechanism would have to be invoked to account for the occurrence of types (iii) and (iv). The interesting genetic studies which are so clearly indicated as a means of clarifying this whole situation have, hitherto, been hindered by the great difficulties encountered in germinating the oospores of most aquatic fungi (cf. GERMINATION OF RESTING SPORES).

Couch (12) and Bishop (4) found indications that diffusible substances were involved in the sexual reactions which they studied in *Dictyuchus* and *Sapromyces*. It remained, however, for Raper (55, 56) to establish beyond doubt, by a brilliantly conceived and executed series of experiments, that there is indeed a regular sequence of reactions under the direct control of a hormonal coordinating mechanism in two gynandromictic species of *Achlya*. He demonstrated conclusively that at least four distinct hormones are involved. Table II summarizes succinctly the action of these substances.

TABLE II

THE ACTION OF HORMONES IN COORDINATING THE SEXUAL PROCESSES OF GYNANDROMICTIC *Achyla* SPS.*

| *Hormone* | *Produced by* | *Affecting* | *Specific Action(s)* |
|---|---|---|---|
| A | ♀-Vegetative hyphae | ♂-Vegetative hyphae | Induces the formation of antheridial branches |
| B | ♂-Antheridial branches | ♀-Vegetative hyphae | Initiates the formation of oogonial initials |
| C | ♀-Oogonial initials | ♂-Antheridial branches | (i) Attracts antheridial branches (ii) Induces, in connection with a thigmotropic response, the delimitation of antheridia |
| D | ♂-Antheridia | ♀-Oogonial initials | Brings about the delimitation of the oogonium by the formation of a basal wall. |

* From Raper (55).

Matings were made in both liquid and solid media. Diffusion of the hormones across cellophane membranes was shown and the sequence of steps and the timing of each was ingeniously demonstrated by many different types of experiments. Reciprocal matings between *A. ambisexualis* and *A. bisexualis* revealed the nonspecificity of certain of the hormones and the specificity of others, the latter providing a precise explanation for the sexual incompatibility of these two species. It was shown that both hormone A and hormone B are stable at 100°C. Intensive studies of the properties and action of hormone A followed (58). It was found that the number of antheridial hyphae

produced was proportional to the concentration of hormone A applied and could be used as an accurate index of reaction intensity. Optimal conditions of temperature, hydrogen ion concentration, and other factors were determined, and standard conditions for bioassay of hormone A were described. In 1942, Raper & Haagen-Smit (60) refined the method of assay still further and succeeded in obtaining, by a complex series of chemical fractionations, a preparation of hormone A 70,000 times more active than the starting material. Unfortunately, despite intensive effort, the substance was not isolated in a pure state, but many of its physical and chemical properties were established. Subsequently, Raper (59a, 59b) discovered that the initial response of the male vegetative plant to the female vegetative plant depends upon a complex of interactions between two substances, A and $A^2$, produced by the female, and two substances, $A^1$ and an inhibitor, produced by the male. The final unravelling of the entire hormonal mechanism in *Achlya* in chemical terms is evidently going to be a difficult task, yet the results achieved thus far deserve great recognition. They have already gone far beyond other similar approaches to an understanding of the mechanisms of sexuality in the fungi and represent the one single instance hitherto of such investigations in the aquatic Phycomycetes.

## Life Cycles and Nuclear Cytology

Outstanding among the recent contributions to our knowledge of the life cycles of the aquatic Phycomycetes are the findings in the order Blastocladiales. In 1930, Kniep (45) had defined the alternation of the two isomorphic generations as we now know them in *Allomyces arbuscula* and *A. javanicus*. Zygotes develop into sporophytic plants which bear resistant sporangia; uniflagellate zoospores from the resistant sporangia form hermaphroditic gametophytic plants which bear male and female gametangia. In 1938, Emerson (20) described a very different cycle in *A. cystogenus* and *A. moniliformis*. In these species there is no evident alternation of generations. Only one type of plant occurs and it is morphologically like the sporophyte of *A. arbuscula*. The spores from resistant sporangia, however, encyst instead of germinating, and each cyst produces uniflagellate swarmers, usually in sets of four. These swarmers, which, as we have seen (cf. Section on SEXUALITY) were later shown by Teter (77) and McCranie (49) to be isogametes that fuse to form zygotes, then give rise to another plant like the parent. Finally, Emerson (21) defined a third cycle in *Allomyces anomalus* in which there is no cyst-formation and no sexuality, the uniflagellate spores from resistant sporangia germinating directly to form plants which again bear resistant sporangia. These three cycles were used by Emerson (21) in 1941 to distinguish the three subgenera *Euallomyces*, *Cystogenes*, and *Brachyallomyces*.

Turning now to the closely related genus *Blastocladiella* we find an extraordinarily parallel series of discoveries. In the first species, *B. simplex*, established in 1937, Matthews (48) described a life cycle which is identical with that of *Brachyallomyces*. A year later, Harder & Sörgel (27) discovered

*Blastocladiella variabilis* and described a sexual life cycle with an alternation of isomorphic generations just like that of *Euallomyces*. Finally, in 1942, in their brilliant monographic studies of *Blastocladiella*, Couch & Whiffen (18) discovered a cyst-forming species, *B. cystogena*, with a life cycle which agrees in every fundamental respect with that of Emerson's subgenus *Cystogenes* as emended by McCranie (49) and Teter (77).

Unfortunately we have scant knowledge of the life cycles of *Blastocladia*, the third genus of the *Blastocladiaceae*, but Blackwell's (6) intensive study of *B. Pringsheimii* indicates that this species should be placed in the Brachyallomyces series.

In 1945 an important contribution was made by Couch (16) when he reported a Cystogenes-type life cycle in *Catenaria Allomycis*, a new species parasitic on *Allomyces*. At the same time, Couch pointed out the parallelism between the life cycle of *C. Anguillulae* and that of *Brachyallomyces*, and on the basis of his keen observations removed *Catenaria* from the Chytridiales and placed it in the family Catenariaceae in the Blastocladiales.

Summarizing all the information on life cycles in the Blastocladiales, obtained by various investigators since Kniep's first demonstration of the alternating generations in *Allomyces javanicus* just 20 years ago, we find the following distribution of species:

Euallomyces cycle: 2 in *Allomyces*, 2 in *Blastocladiella*
Cystogenes cycle: 2 in *Allomyces*, 2 in *Blastocladiella*, 1 in *Catenaria*
Brachyallomyces cycle: 1 in *Allomyces*, 3 in *Blastocladiella*, 1 in *Catenaria*, 1 in *Blastocladia*.

This list includes the species treated by Emerson (21), Couch & Whiffen (18), Couch (16), and Blackwell (6), as well as Whiffen's (85) cyst-forming *Blastocladiella microcystogena*.

The basic significance of these life-history studies in the aquatic Phycomycetes has recently been brought into sharp focus by the work of Emerson & Wilson (24), on meisosis in *Allomyces*. The extended controversy regarding nuclear behavior in this genus is reviewed in their paper and was discussed at some length by Emerson (21) in 1941. Suffice it to say that the place of meiosis in the life cycle of *Euallomyces* was not certain, and nothing at all was known of its position in *Cystogenes*. With aceto-orcein preparations, Wilson demonstrated beyond doubt that the reductional chromosome divisions occur in both *Euallomyces* and *Cystogenes* when the resistant sporangia germinate. Detailed observations were made of first and second meiotic figures and it was established that these two nuclear divisions are the only ones which occur in the resistant sporangia. Thus, it is now clear that the sporophyte of *Euallomyces* is diploid and the gametophyte is haploid, while in *Cystogenes*, all parts of the life cycle are diploid with the exception of the spores from resistant sporangia, the gametangial cysts, and the gametes.

The diploid plants of *Euallomyces* and *Cystogenes* reproduce vegetatively by uninucleate, uniflagellate zoospores from thin-walled zoosporangia. In *Euallomyces* these swarmers are morphologically indistinguishable from the uninucleate, uniflagellate zoospores released from resistant sporangia.

For want of a better basis of distinction, Emerson (21, p. 89) applied the following names to the two types of spores: those from thin-walled zoosporangia were called "zoospores," those from resistant sporangia were called "R. S. zoospores." It is now evident that the two sorts of spores can be named in accordance with the fundamental differences which exist in their origin and chromosome number. The nuclei in spores from resistant sporangia arise directly by meiosis and have the haploid number of chromosomes, those from thin-walled zoosporangia arise by mitosis and have the diploid number. The haploid zoospores of *Allomyces*, therefore, fall in the general category of "meiospores," as defined by Wahl (79), and the writer proposes that the parallel term "mitospore" can very appropriately be applied to the diploid zoospores of *Allomyces*.

Although cytological evidence is still lacking, it seems highly probable that meiosis occurs in the resistant sporangia of all of the other genera and species in the Blastocladiales which have life cycles like those of *Euallomyces* or *Cystogenes* (see above). We appear, then, to be combining in this order a whole group of aquatic fungi which are sharply distinguished from all other eucarpic types by the following fundamental features of their life cycle: (*a*) the zygote germinates immediately without a rest period; (*b*) the vegetatively reproducing plant is a true diploid which forms diploid mitospores; (*c*) the resting stage is an asexually formed diploid resistant sporangium; (*d*) meiosis occurs when the resistant sporangium germinates. On the other hand, the life cycle which, as far as we know, characterizes the great majority of sexually reproducing aquatic Phycomycetes outside of the Blastocladiales is haplontic. In this cycle: (*a*) the zygote does not germinate immediately since it enters into a resting condition; (*b*) the vegetatively reproducing plant is haploid and forms haploid mitospores; (*c*) the resting structure is the transformed zygote; and (*d*) meisosis probably occurs when the zygote germinates. In his treatment of life cycles in the algae, Fritsch (26) has expressed the view that the haplontic type is primitive, while isomorphic cycles, like that of *Euallomyces*, are probably derived. The diplontic cycle, like that of *Cystogenes*, is also a more advanced type which may have arisen directly from a haplontic type or come indirectly by gradually increased dominance of the sporophyte in an originally isomorphic cycle. Whatever future interpretations may be put on these questions in the Phycomycetes, we should certainly recognize fully now that the demonstration of diploidy in *Allomyces* has revealed an area of knowledge in these fungi that must play an important role in determining their natural relationships.

One illustration may serve to emphasize this point. Kniep's (44) discovery of sexuality in *Allomyces* was hailed (27, 44, 80) as a link that had long been sought for in the uniflagellate series of Phycomycetes. Here was a form with unequal motile gametes that beautifully bridged the gap between isogamous chytridiaceous fungi and Cornu's strikingly oogamous *Monoblepharis*. Now, in the light of our knowledge of the life cycle of *Allomyces*, any sort of direct evolutionary line from the chytrids through *Allomyces* to *Monoblepharis* hardly seems tenable. Though better cytological evidence is called for,

*Monoblepharis* is almost certainly, like all the higher members of the biflagellate series, a haplontic organism in which the first divisions of the zygotic nucleus are meiotic. It is hard to conceive of such an organism evolving from an ancestor with an isomorphic cycle or a diplontic cycle such as those of *Euallomyces* or *Cystogenes*. The progenitor of *Monoblepharis* ought to be a haplontic fungus with anisogametes, and it may well be that such a genus will be discovered at some future time.

We might conclude that there appear to be two major evolutionary lines of development in the uniflagellate Phycomycetes, both derived from chytridiaceous ancestors, one haplontic and leading to *Monoblepharis*, the other first isomorphic then diplontic and leading to *Allomyces*. Even this, however, may be a bad oversimplification. The cyst-forming species of *Allomyces* are isogamous, and it would be contrary to fundamental concepts of the evolution of sexuality to derive isogamous *Cystogenes* from anisogamous *Euallomyces*. Moreover, even in *Blastocladiella*, which is almost as primitive as a monocentric chytrid in its thallus form, we find species probably representing the diplontic cycle as well as the isomorphic. Moreover, while the diplontic cyst-formers here are isogamous, just as are their counterparts in *Allomyces*, one of the species which shows an isomorphic (Eual-lomyces-type) life cycle has gametes which differ in pigmentation (cf. Section on SEXUALITY) and can be considered anisogamous. There are, therefore indications that the isomorphic and diplontic species of *Allomyces* may have evolved from isomorphic and diplontic forms of *Blastocladiella*, respectively. If this were true we would have to recognize at least three, rather than two, lines of evolution from chytridiaceous forms to higher types in the uniflagellate series, a haplontic line, an isomorphic line, and a diplontic line.

A few words about life cycles in the Chytridiales themselves will justify to some extent what might otherwise appear to be idle speculations. While a fair amount of information concerning the variety of sexual mechanisms occurring in the chytrids has been accumulated (71), our knowledge of their life histories in terms of chromosome behavior is still in a very nebulous state. Two basic patterns of development have been recognized however; in one gametic fusion immediately precedes the development of resting structures; in the other resting structures are formed asexually. As a working hypothesis it would seem highly worthwhile to consider the likelihood that the organisms in the former group are haplontic types like *Monoblepharis*, while those in the latter group are isomorphic, like *Euallomyces*, or diplontic, like *Cystogenes*. At least the systematic testing of such a hypothesis should provide information which would greatly clarify our entire concept of the reproductive processes and phylogenetic relationships of the primitive aquatic fungi. Furthermore, the stage is well set for a concerted program of research along these lines. Means are at hand for controlled pure culture work with the Chytridiales (cf. THE USE OF PURE CULTURES). Single spore studies can be carried out and the requisite conditions for controlled production and germination of the resting structures can be established (cf. Section on GERMINATION OF RESTING SPORES). And the simple and effective manner in

which modern staining techniques can be applied to the aquatic Phycomycetes has been amply demonstrated (24).

Before concluding this discussion, a few further comments should be added concerning deviations from the normal life cycle which characterizes *Euallomyces*. It has been repeatedly shown (21, 31, 68) that in certain isolates of *Allomyces arbuscula* and *A. javanicus* the zoospores from resistant sporangia may, more or less regularly, develop into sporophytic plants directly. From an interesting series of tests which they made, Hatch & Jones (31) concluded that this "short-cycling" was related to two factors: the length of time the resistant sporangia were dried and the concentration of nutrient provided for the germinating R. S. zoospores. Drying for six days followed by germination in standard nutrient gave nearly 90 per cent sporophytes, whereas, drying six days followed by germination in 1/50 strength nutrient or drying for 24 days followed by germination in standard nutrient gave 100 per cent gametophytes. As yet we do not know what chromosomal adjustments occur in *Euallomyces* when R. S. zoospores form sporophytes directly or when other deviations, such as parthenogenetic formation of sporophytes (21, 68), occur. It is very likely, however, that future work on this problem will reveal fundamental information about some of the factors controlling the alternation of generations and meiosis in *Allomyces*.

The occurrence of "short-cycling" in *Euallomyces* gives us some clue as to the significance of the life cycle in *Brachyallomyces*. The writer (21) recognized in 1941 that *Brachyallomyces*, with its one species *A. anomalus*, was being "tentatively established to include those few isolates in which repeated attempts to obtain sexual plants have been unsuccessful." Actually, only two of the writer's 51 isolates were listed under *Brachyallomyces*, and since that time both of them have revealed a sexual stage and been transferred to *Euallomyces*. Although there are strong indications, therefore, that persistent study will ultimately demonstrate a gametophytic stage in nearly all non-cyst-forming isolates of *Allomyces*, the short cycle may have become fixed in a few strains. The growing number of other species of the Blastocladiales (see above) which appear to exhibit this cycle also indicates the likelihood of its reality, but as with *Allomyces anomalus*, continued efforts should be made to show whether sexuality has been overlooked in these forms or whether it truly does not occur. Presumably those members of the Blastocladiales which have the Brachyallomyces cycle are diploid, having been derived from closely related isomorphic species by suppression of meiosis and loss of the gametophyte.

## Genetics and Cytogenetics

Although the aquatic Phycomycetes offer some very interesting possibilities for genetic research, little work of this sort has been published to date. Couch (12) reported crosses between different strains of *Dictyuchus monosporus* in 1926. Between 1936 and 1940 similar inter-strain matings were described by Weston (81) and Bishop (4) in *Sapromyces elongatus* and by Raper (53, 57) in *Achlya bisexualis* and *A. ambisexualis*. Salvin (63) described

some intergeneric crosses between *Thraustotheca primoachlya* and *Dictyuchus achlyoides* in 1942. These studies contributed very significantly to our knowledge of sexual mechanisms (see SEXUALITY) and systematic relations (see EXPERIMENTAL TAXONOMY), but in none of them was anything approaching a complete genetic analysis made. For several reasons the Saprolegniaceae, Leptomitaceae, and Pythiaceae are rather unsuited for genetic work. The gametes of both sexes are non-motile and are not released from the gametangia. Hence, crosses can only be made when two thalli are brought into close contact, and the problem is further complicated by the fact that the great majority of species are homothallic and self-fertile. Apomixis is also a very common phenomenon. And finally, even when authentic crosses have been effected, great difficulty has usually been encountered in germinating the hybrid oospores.

Prospects for experimental hybridization and genetical analysis are much better in the uniflagellate Phycomycetes where uninucleate, motile gametes are released from the gametangia and can readily be used for making crosses. From the foregoing discussions of resting spore germination, sexuality, and life cycles, it will be apparent that the species of *Euallomyces* provide exceptionally favorable material for fundamental genetic research. In 1941, Emerson (21) reported very briefly the results obtained and the conclusions drawn from an extensive series of interspecific reciprocal hybrids between *A. arbuscula*, which has the hypogynous arrangement of gametangia, and *A. javanicus* var. *macrogynus*, which has the epigynous arrangement (cf. page 181). Since the place of meiosis in *Euallomyces* was very much in doubt at that time, Emerson hoped to discover by genetical means where segregation and the reduction divisions occurred. He summarized his results as follows [(21) p. 98]:

> (1) Gametophyte plants arising from any single $F_1$ sporophyte, obtained from a single zygote, are of many different sorts, i.e., both parental types, 100% hypogynous and 100% epigynous, segregate out, as well as a series of intermediates ranging from types which show nearly pure epigyny to those showing nearly pure hypogyny. (2) An exactly similar series of gametophytes can be obtained from any secondary $F_1$ sporophyte started from a single zoospore [i.e. mitospore] discharged from a zoosporangium borne on a primary $F_1$ sporophyte.

Emerson drew two main conclusions from these results: "the arrangement of gametangia is a quantitative character controlled by polymeric genes" and "reduction divisions and segregation of the parental characters take place after the formation of resistant sporangia on the sporophyte and before the development of mature gametophytes."

The recent cytological demonstration (24) of meiosis in *Allomyces* served not only to bear out these conclusions but also to reveal a very interesting series of cytogenetic phenomena. Work on this phase is still in progress and detailed reports of all the genetical and cytological observations are in preparation, but certain of the main results merit inclusion in the present discussion. It is now clear that *A. arbuscula* and *A. javanicus* var. *macrogynus* have different haploid chromosome numbers. These were first reported

(24) as 7 and 14, respectively. Counts were based on what were then interpreted as pressed out anaphase figures of meiosis I, showing 14 and 28 chromatic bodies in the nuclei. More critical analysis of such figures has shown that they actually represent a pre-metaphase state in which the paired condition of the chromosomes can now be recognized and demonstrated in photomicrographs. Furthermore, one additional pair of small chromosomes was discovered in *A. arbuscula.* The correct haploid numbers appear, therefore, to be 16 in *A. arbuscula* and 28 in *A. javanicus* var. *macrogynus.*

Meiotic divisions were studied in the $F_1$ resistant sporangia and provided the key to a sound understanding of the hybridization experiments. Complete details have not been worked out yet, but it is evident that variable and incomplete pairing occurs and results in random distribution of many of the chromosomes. This unquestionably accounts for the very low ($<0.3$ per cent) viability of the meiospores from the $F_1$ and also provides a reasonable explanation of the chromosome counts in the $F_2$ generation. $F_2$ sporophytes are obtained by selfing individual $F_1$ gametophytes. Pairing of the chromosomes at the next ($F_2$) meiosis is essentially complete and regular, since the sets of chromosomes contributed by the male and female gametes are identical, and viability of the meiospores returns to the high level ($>50$ per cent) found in the parental species. Different $F_2$ strains, however, have different chromosome numbers: one hypogynous strain shows 32 pairs at meiosis, four intermediate types show 20, 26, 27, and 42 pairs, respectively, and an epigynous strain shows 27 pairs. Apparently there is no direct relation between chromosome number and gametangial arrangement, epigyny or hypogyny being expressed as a result of the total balance between E and H genes present in the genotype.

Because of their similarity to certain of the experimental hybrids, Emerson (21) suggested in 1941 that *A. javanicus* var. *javanicus* and var. *perandrus* might be natural hybrids. In both of these varieties the epigynous arrangement of gametangia is predominant but a small percentage of hypogynous pairs occurs. The discovery of 19 and 21 pairs of chromosomes at meiosis in certain isolates of these varieties strengthens the belief that they have indeed evolved through a process of hybridization much like that which has just been described. Interesting further evidence on this problem has come from nutritional studies now being carried out by John L. Ingraham in the writer's laboratory. In the course of a routine check to determine whether all species and varieties of *Allomyces* have the same vitamin requirements, it was found that an isolate (Java 1) of *A. javanicus* var. *javanicus* was the only one which was heterotrophic for any vitamin except thiamine. While it could be assumed that a natural mutation involving the mechanism for synthesis of the required vitamin had occurred, another possibility was presented by the presumed hybrid origin of Java 1. In the random distribution of chromosomes which takes place at the first meiosis, following hybridization of *A. javanicus* var. *macrogynus* and *A. arbuscula,* whole chromosomes, carrying genes responsible for certain synthetic capacities, must be lost. If both the paternal and maternal chromosomes carrying a particular gene were

lost and the resulting deficiency were not lethal, it would appear in the hybrid. Following this hypothesis other putative natural hybrids, as well as several of the artificial hybrids, were tested and deficiencies in synthetic capacity were found in about 50 per cent of them. Aside from their bearing on the problem of hybridity in *Allomyces*, these studies suggest that nutritional deficiencies may evolve in nature quite aside from gene mutations.

Some induced deficiencies have been produced in *Euallomyces* by treatment of meiospores with ultraviolet irradiation [Yaw (87)] and mitospores with nitrogen mustard (Machlis, unpublished work at the University of California). Yaw obtained an arginineless strain (A) and a lysineless strain (L) of *Allomyces arbuscula*. She has reported that typical dominance relations are shown in the sporophytes from crosses of A×L and in those from back crosses to the wild type, but genetic analyses have not yet clearly established the one-gene nature of the mutant characters.

Undoubtedly genetic research with the aquatic Phycomycetes can contribute materially to an elucidation of fundamental problems in this field of biology, and it is to be hoped that experimental studies of inheritance in other genera of the Blastocladiales will be undertaken in the near future.

## Experimental Taxonomy

While emphasis in the foregoing pages has been placed on the cultural and experimental aspects of current research in the aquatic Phycomycetes, it will be apparent that many phases of these studies are bound to have a fundamental bearing upon our concepts of phylogeny and systematic groupings. Modern taxonomic work on the water fungi must, in so far as possible, be based upon pure cultures in which the morphological variability of single-spore isolates can be established under controlled conditions, where reproductive processes and life cycles can be fully worked out, where genetic relationships can be established, and where physiological characteristics of the orders, families, genera, and species can be determined. If we will only recognize it, we are confronted by a most interesting prospect of productive work in experimental taxonomy. The remaining paragraphs will be devoted, therefore, not to a survey of the many new species and genera that have appeared in the literature of the past decade, but rather to a review of certain kinds of studies which illustrate so well the nature of the main task before us.

*Variability.*—Although the great variability of form and behavior of the water fungi has become quite generally recognized in an abstract sort of way, all too few investigators of the taxonomy of this group have paid serious attention to it in their studies. There appears to be a greater fascination in describing new species than in determining the precise limits of their variability and the causes thereof, whether genetical or environmental. Research of the latter type, though arduous and time-consuming, is urgently needed and will be of the greatest overall importance. It seems high time to recognize the responsibilities involved in naming new species: the job of critically establishing the true extent of variability and evaluating species and generic limits cannot be postponed indefinitely for others to undertake

in the distant future. The amazing gaps which exist in our knowledge of this aspect of the aquatic fungi have led already to a degree of confusion which is becoming ever more serious, particularly in such major groups as the Saprolegniales and Chytridiales.

Salvin's (61, 62) experimental studies of the Saprolegniaceae, published in 1942, provide an outstanding example of the sort of work that needs to be done and the valuable results which can be obtained. By varying temperature, aeration, and the volume of water used, Salvin demonstrated that a single-spore strain of *Brevilegnia* exhibited, under different environmental conditions, the characteristics of five putatively different species. He concluded further that the genus *Brevilegnia* itself could not be clearly distinguished from *Thraustotheca* and should be reduced to synonymy. His studies of *Dictyuchus achlyoides* and *Thraustotheca primoachlya* revealed that an organic product of metabolism which accumulates in the medium is responsible for the change from the achlyoid behavior of spores in young cultures to the dictyuchoid or thraustothecoid behavior subsequently. Such findings point up the whole problem of generic limits in the Saprolegniaceae and emphasize the dubious advantages of labelling each intermediate type as a new species or even genus. Just how are we to interpret an entity like *Achlya dubia*, for instance, described in 1923 in the following terms (11, p. 135): "discharging the spores as in *Achlya* or as in *Thraustotheca* in varying proportion, often about half and half on a termite in sterilized well water, not rarely behaving as in *Dictyuchus*"? No one who has worked with this group will doubt that these and many other intergrading types occur, but the question to decide is how they arose and how they should be dealt with in a significant and usable taxonomic scheme. Salvin (63) considered the possibility, which seems highly likely, that they have resulted from natural hybridization. Unfortunately, although intergeneric matings between *Achlya flagellata* and *Thraustotheca clavata* were apparently successful, the final experimental demonstration of generic intergrades was not achieved, since the hybrid oospores could not be induced to germinate.

With increased knowledge of the factors concerned in oospore maturation and germination (see Germination of Resting Spores) the prospects for successful genetic studies are improved, making it possible to follow up Salvin's pioneering efforts. Considering the ease with which most members of the Saprolegniaceae can be isolated and maintained in pure culture, it is surprising that there are as yet no generic monographs involving the use of large numbers of isolates compared under a wide variety of conditions and tested for their genetic relationships. Middleton's (50) masterly treatment of the genus *Pythium* in the Peronosporales provides a striking contrast in which the taxonomy of the 66 valid species is based upon a biological study of over 2,000 isolates grown and compared in different environments in pure cultures.

The Chytridiales offer a more difficult problem, but one which is fully as urgent. New species and genera are presently being added to this large order at a rapid rate as a result of widening interest and the application of

special collecting methods in which particular types of substrata are being employed (see pp. 171–73). That questions of overlapping and intergradation are becoming increasingly acute is evident from numerous statements occurring in recent taxonomic works. Reference to Sparrow's treatment of the Chytridiales in 1943 (71) shows that such features as the type of rhizoidal system, the presence or absence of an apophysis, the monocentric or polycentric nature of the thallus, and the relation of the thallus to the substratum are used as key characters to distinguish many of the families and genera. Yet in 1945 Karling (37) concluded that "the presence of an apophysis and the nature of the rhizoids" in certain species he was considering "are variable characters and of doubtful importance in distinguishing genera." Again in 1947 (39) he pointed out that his new species, *Phlyctorhiza variabilis*, showed some of the characters of *Rhizophlyctis* as well as those of Hanson's new genus *Phlyctorhiza*. And finally, in 1949 (42), in his recent diagnosis of *Rhizidium varians* as a new species, he remarked that it "combines the structural and developmental characteristics of a number of genera and even families"! After describing *Septochytrium variabile* as a new genus and species in 1939, Berdan (3) reported in 1942 an interesting series of observations on the variability of a single spore strain which led her to state that "so many variations occur in the development of this chytrid that it is very difficult to describe any one type as definitely representing the normal." Although it is "predominantly polycentric" it is also "often monocentric"; the sporangia are "rarely apophysate." She concluded that the data obtained "may suggest some evolutionary connection between the genus *Endochytrium* of the Rhizidiaceae and the operculate genera of the Cladochytriaceae" and that "certainly the limits of the two families do not seem to be quite so sharply defined as previous diagnosis might lead us to believe." In 1944 Whiffen (84) questioned several of the main bases of systematic groupings in the Chytridiales. The validity of her argument against the fundamental distinction between operculate and inoperculate forms can be questioned, but her objection to assigning eucarpic monocentric chytrids to families on the basis of the relation of the thallus to the substratum is certainly sound. It remains to be seen whether the five types of thallus development, by which she proposed to distinguish the main groups of eucarpic Chytridiales, are sufficiently fixed to be of practical use and are sufficiently basic to show natural relationships.

Without laboring the point any further it should be evident that many of the morphological features currently used as key characters in the Chytridiales are highly variable, and that, just as in the Saprolegniales, monographic studies are critically needed. This has been prevented in the past largely by the difficulty of obtaining and maintaining single-spore isolates, but with the ready methods of pure culture now known, it should be entirely feasible to build up large collections for comparative investigation and we may look forward to a period of controlled study of this fascinating group of lower Phycomycetes. We can also be sure that sounder bases for taxonomic groupings will become evident as more information about the sexual proc-

esses and life cycles of the chytrids and other primitive forms becomes available.

An interesting contrast between *Blastocladia* and *Allomyces* in the Blastocladiales will serve well to conclude this discussion of variability. There are about 12 described species in the former genus based, for the most part, upon subtle differences in the form and dimensions of the thalli and sporangia. Lloyd (47) has described the great range of variability in naturally-occurring plants of the type species, *B. Pringsheimii*, and the writer has repeatedly noted the markedly different types of thalli which develop in single spore cultures of this same species grown under different controlled environmental conditions. It is probable, therefore, that about half of the presently accepted species are invalid. On the other hand, only five species of *Allomyces* were recognized in Emerson's (21) treatment of this genus in 1941. Some 50 isolates, gathered from widely different localities around the globe, were compared under defined conditions in pure culture. A systematic study of the range of intra- and inter-strain variability revealed a reticulate pattern of intergradation of most of the morphological characters of the sort generally used to distinguish species of water fungi. The results showed conclusively that such characters had no significance when a sufficiently large population was analyzed. Further, the probable occurrence of natural interspecific hybridization was established (see GENETICS AND CYTOGENETICS) and its significance in the taxonomy of the genus was recognized. Noteworthy is the fact that in the nine years which have elapsed since the publication of this monograph, despite frequent subsequent reports of *Allomyces* from many parts of the world by numerous investigators, no new species have been described.

*Host-specificity.*—There has been a general tendency in the past to assume, especially in the Chytridiales and Lagenidiales, that parasitic forms found on new hosts should be assigned new specific names even when few, if any, morphological differences exist. A particularly vivid example is provided by the holocarpic genus *Rozella* whose 12 or more species have been defined largely on the basis of host-specificity despite the paucity of evidence based on cross-inoculation experiments. It is encouraging to find that more critical attention is being devoted to this problem in current studies. Eminently interesting in this connection are investigations such as those of Karling (34) and Shanor (67) on *Rozella*, Karling (40) on *Olpidium*, Couch (16) on *Catenaria*, Couch *et al.* (17) on *Octomyxa*, and Shanor (66), Whiffen (83), and Karling (41) on *Olpidiopsis*. Efforts to determine the host ranges of these organisms lend much greater credence to the conclusions reached regarding host specificity and show that only after much further similar work can host relations be seriously considered in taxonomic groupings of these and other parasitic aquatic fungi. Even in those instances, however, where limited host ranges are clearly demonstrated, they should, in the writer's opinion, be used only as varietal characters and not to distinguish species. Perhaps it is not too much to hope that pure two-membered cultures, like those of *Rozella* and *Woronina* noted in the section on THE USE

of Pure Cultures (p. 170), may one day permit us to discover sexuality in these parasites and determine the degree of genetic relation which exists between different host-specific strains.

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# THE DEVELOPMENT OF BACTERIAL RESISTANCE TO CHEMOTHERAPEUTIC AGENTS[1]

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This review is limited to the literature on the development of resistance by bacteria to the sulfonamide drugs and the antibiotics in general use today.[2] As each of these chemotherapeutic agents has become available for experimental and clinical investigation, a series of analogous observations has been made. First, it was noted that within each of the susceptible species of bacteria, there were minor but detectable differences among the various strains. It was then observed that resistance in a susceptible strain could be acquired (*a*) *in vitro* during serial cultivation in media containing increasing concentrations of the drug, or (*b*) *in vivo* during repeated passage through animals treated with subcurative doses of the drug. In addition, the clinical observation was made that failure of therapeutic response in subacute or chronic infections in man might be associated with development of resistance during the course of treatment.

Two general theories have been advanced to explain the emergence of resistant strains: (*a*) that they are the result of adaptation by the bacterial cell, a process which is induced by the direct action of the drug, and (*b*) that they arise from resistant individuals which appear in the bacterial population as the result of spontaneous mutation. This latter theory has gained wide acceptance. It assumes that the drug plays no direct role in the process, but serves only as a selective agent which prevents the growth of all members of the bacterial population except such mutants as are resistant to its action.

## The Sulfonamide Drugs

*Experimental studies.*—The first observations on sulfonamide resistance were made by MacLean, Rogers & Fleming (3), who noted differences in sensitivity to sulfapyridine among strains of pneumococci isolated from patients. They also found that a sensitive strain of pneumococcus could acquire resistance to sulfapyridine by passage through mice treated with subcurative doses of the drug.

MacLeod & Daddi (4) demonstrated the development of resistance *in vitro* by cultivating a strain of pneumococcus repeatedly in broth containing

[1] This review covers approximately the period from 1939 to December, 1949.

[2] It should be noted as a matter of historical interest that at the very beginnings of modern chemotherapy, Ehrlich (1) and his collaborators, Browning (2), Franke, and Röhl observed that trypanosome infections in mice which had survived inadequate treatment became resistant to full therapeutic doses of atoxyl and of certain dyes which were then under investigation.

sulfapyridine. The resistance thus acquired was demonstrable not only *in vitro*, but also *in vivo*. It was not associated with any alterations in morphology, virulence, or specific immunological properties. Other reports described the development of resistance by pneumococci to all sulfonamide derivatives both *in vitro* and *in vivo* (5 to 8).

Further studies on acquired resistance by pneumoccoci to sulfanilamide, sulfapyridine, and sulfathiazole showed that a strain which had become resistant to one drug was resistant to the other two as well. The rate at which resistance developed to any one drug differed from strain to strain, and the rate for any single strain differed from drug to drug. In general, it proceeded most rapidly with the least effective drug and most slowly with the most effective; i.e., the rate bore a reciprocal relationship to the effectiveness of the drug (9). The observation that strains resistant to one of the sulfonamide drugs were also resistant to other derivatives was reported for the gonococcus (10) and *Escherichia coli* (11).

Additional reports soon appeared describing the development of sulfonamide resistance *in vitro* by a variety of microorganisms; e.g., most of the *Neisseriae* (12), *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) (13), the gram negative bacilli (14, 15), *Mycobacterium ranae* (16), and the viruses of lymphogranuloma venereum (17) and psittacosis (18).

*Clinical observations.*—The first clinical report of the development of resistance during the course of treatment in man seems to have been made by Ross (19) who described such a change in strains of pneumococcus recovered from time to time during a fatal case of meningitis treated with sulfapyridine. This report was soon followed by those of Lowell *et al.* (5) and Auger (20) who described similar therapeutic failures as a result of the appearance of resistant organisms. Progressive increase in resistance was observed by Hamburger *et al.* (21) in strains of pneumococci isolated from a case of endocarditis treated for six months with sulfapyridine. Other instances of the development of resistance during the course of pneumococcal (22, 23) and staphylococcal (24) infections have been reported.

A few years after the sulfonamide drugs came into general use, evidence began to appear suggesting that their widespread clinical administration had led to an increase in the resistance of certain strains of bacteria. The two infections which attracted particular attention were those caused by gonococci and by Group A hemolytic streptococci, although a similar occurrence was observed in infections caused by staphylococci (25). Among cases of acute gonococcal infection, there occurred a marked increase in the percentage which failed to respond to treatment. As these infections were demonstrated to be caused by sulfonamide-resistant strains, it was apparent that such strains had become more prevalent (26 to 30).

Either of two explanations of these findings is possible: (*a*) that individual strains of the gonococcus had each acquired resistance *in vivo* during exposure to the drug in its host, or (*b*) that the widespread use of the drug had eliminated many sensitive strains already present. Among a number of observations supporting the latter hypothesis is that of Schmith & Reymann (31)

who found some sulfonamide-resistant strains among 50 old laboratory cultures which had been isolated from patients in the presulfonamide era.

A more striking instance of the increased prevalence of resistant strains associated with the use of sulfonamide drugs occurred during World War II at certain training centers of the U. S. Navy (32, 33). In an effort to reduce the incidence of scarlet fever and other streptococcal diseases, prophylactic doses of sulfadiazine were adminstered to large numbers of men. This program of mass chemoprophylaxis was successful for a few months, but was followed by a rapid increase in the incidence of infection with drug-resistant strains of Group A hemolytic streptococci, most of which belonged to Types 3, 17, or 19. The question arose and has been much discussed whether the emergence of these strains was a direct result of the widespread prophylactic administration of the drug (34). This possibility cannot be ruled out and is supported by the fact that at one station no resistant strains were encountered among 40 isolated before the trial was initiated (33). However, the size of this sample was too small to exclude the possibility that a few resistant strains were already present in the population. A somewhat more convincing argument can be made for the supposition that the administration of the drug merely eliminated the sensitive strains from the treated populations and that pre-existent drug-resistant strains were responsible for the epidemic.

This latter explanation is supported by the paucity of experimental evidence that Group A streptococci can acquire resistance *in vitro*. There is, to be sure, the report of Cutts & Troppoli (35)that a strain already resistant to 5 mg. per cent of sulfanilamide increased its resistance to 74 mg. per cent and that of Chandler & Janeway (36) which mentions that a culture transferred repeatedly in sulfonamide-containing broth became more resistant to the bactericidal effect of additional amounts of the drug. There is also the case of bacterial endocarditis in a horse caused by a Group A streptococcus which developed resistance during treatment with sulfonamide (37).

However, Coburn & Wilson both failed in numerous attempts to increase resistance in a large number of strains by repeated subcultivation in a variety of media containing subinhibitory concentrations of sulfonamide (38). Careful consideration of all the evidence available leaves one still in doubt about the precise role played by the prophylactic administration of sulfadiazine in the epidemic which followed.

Another epidemiological study which should be mentioned was made on a large group of rheumatic children who received sulfanilamide for an average of 2.5 years. It was found that in the course of this treatment, the incidence of Group A hemolytic streptococci as determined by frequent throat cultures was significantly reduced, but not eliminated. However, 29 per cent of the Group A strains isolated from throats were found to be resistant to 10 mg. per cent of sulfanilamide as compared with 6 per cent among the strains isolated from a similar group of untreated controls (39).

There can be little doubt that more work is needed on the effect of prolonged administration of antibacterial drugs on the susceptibility of the re-

spiratory tract to infections with drug-insensitive bacteria. It is possible that alteration of the normal flora resulting from such medications may not always be wholly beneficial.

*Biochemical changes involved in sulfonamide resistance.*—The presence in bacteria of a substance which inhibits the action of the sulfonamides was first reported by Stamp (40). From broth cultures of a Group A and a Group C streptococcus, he isolated and described the properties of a fraction which antagonized both sulfanilamide and sulfapyridine. Green (41) also concluded that some bacteria contained a substance which interfered with the action of sulfanilamide because large inocula could initiate growth in concentrations of the drug which inhibited the growth of small inocula. This factor was found to be increased in strains which had survived for some time in the presence of sulfanilamide.

MacLeod (42) found that the sulfonamide inhibitor was greatly increased in a strain of pneumococcus as the strain acquired sulfapyridine fastness by repeated cultivation in the presence of the drug. Tillett *et al.* (43) confirmed MacLeod's observation on the same strain of pneumococcus but were unable to demonstrate sufficient inhibitor to explain the resistance of two other strains isolated from cases which were refractory to sulfonamide therapy.

Woods' attempt (44) to isolate the sulfonamide inhibitor in yeast led him to the discovery that *p*-aminobenzoic acid (PAB) was a powerful antagonist of sulfanilamide. This observation was of great importance because it resulted in the formulation of an explanation of the action of antibacterial agents, now known as the Woods-Fildes theory. Fildes (45) had already suggested that antibacterial substances may operate by interfering with some essential metabolic process in the cell. The molecular similarity of sulfanilamide to PAB, which is a necessary growth factor, led Woods and Fildes to the conclusion that sulfanilamide prevented the growth of bacteria by blocking some essential reaction involving PAB.

Although increased production of PAB accounted in some instances for acquired sulfonamide resistance (46, 47, 48), it has not been found to occur in all instances (49, 50, 51) and is, therefore, not the sole mechanism whereby resistance can develop. Emerson (52) has described a mutant strain of *Neurospora crassa* which requires sulfanilamide for growth and is inhibited by PAB. A double mutant requires for growth both sulfanilamide and PAB in proper concentrations.

The following changes in biological activity were reported by MacLeod (53) in sulfonamide-resistant strains of pneumococci: marked diminution in hydrogen peroxide production and in dehydrogenase activity for glycerol, lactate, and pyruvate, but not for glucose. Clapper & Heatherman (54) found reduced production of hydrogen peroxide but increased dehydrogenase activity for glycerol, lactate, pyruvate, and also glucose in a resistant strain of an alpha hemolytic streptococcus and concluded that the mechanism for the development of sulfonamide resistance in streptococci differed from that of pneumococci.

### PENICILLIN

*Experimental studies in vitro.*—The first comprehensive report on penicillin in 1941 by the investigators at Oxford (55) included the observation that the concentration of penicillin required for inhibition of a strain of *M. pyogenes* var. *aureus* increased 1,000-fold during 16 weeks' cultivation in broth containing increasing concentrations of the drug. Since then, a number of papers have described the development of penicillin resistance *in vitro* by a variety of bacteria; e.g., staphylococci (56 to 64), pneumococci, (58, 59, 65), streptococci (58, 59, 65 to 69), meningococci (70, 71), gonococci (72, 73, 74) and *Actinomyces bovis* (75).[3]

The rate at which resistance was acquired and the degree of resistance varied from strain to strain depending, presumably, upon the rate of mutation. Certain strains of staphylococci had the fastest rate, and most strains of Group A hemolytic streptococci, the slowest, some of the latter failing altogether to acquire resistance.

*Permanence of acquired resistance.*—There has been considerable disagreement on the permanence of resistance acquired *in vitro*. In view of the fact that most strains which have acquired resistance multiply more slowly than the sensitive strains from which they were derived, it is not surprising that a sensitive cell arising in a resistant culture by backmutation could overgrow the rest of the population in a drug-free medium. Miller & Bohnhoff (71) found the resistance of meningococci to diminish at approximately the same rate at which it was acquired. Both changes doubtless depend upon the mutation rate of the strain.

Most investigators who have studied the development of resistance in *M. pyogenes* var. *aureus* have found it to be gradually lost during repeated subcultivation in penicillin-free media (60, 62, 63, 64, 77). An exception was a highly resistant strain which showed marked alterations in morphological and biochemical characteristics when grown in high concentrations of penicillin and also retained its resistance when transferred repeatedly in penicillin-free broth (78).

Graessle & Frost (63) observed that three of five strains of *M. pyogenes* var. *aureus* which had acquired low degrees of resistance became more sensitive during 9 to 12 months' storage in the dry-ice refrigerator. This was also the experience of Miller & Bohnhoff with their strains of resistant meningococci and gonococci (79).

On the other hand, strains of pneumococci were found to retain their re-

[3] The occurrence of resistant mutants in penicillin-containing media should not be confused with the survival of "persisters," first described by Bigger (76). He found that failure to sterilize a broth culture of *Staphylococcus* was due to the presence of a few cells which had withstood the effect of a concentration of penicillin lethal to the rest of the bacterial population. The persisters are thought to survive because they happen to be in a resting state at the time of their exposure to penicillin. They are not resistant mutants for their progeny are as sensitive as the original culture.

sistance after repeated subcultivation in drug-free media (58, 59, 65), and two reports indicated that acquired resistance was maintained by three strains of *M. pyogenes* var. *aureus* (58) and one strain of *Streptococcus hemolyticus* (65). Although strains of *S. pyogenes* (59) and hemolytic streptococci of Groups A and C (67, 69) lost their acquired resistance, Gezon found that 10 strains of Group B (68) streptococci retained it during growth on control media.

By careful examination of naturally resistant penicillinase-producing strains of *M. pyogenes* var. *aureus* Barber (80) was able to detect the appearance of sensitive variants after prolonged cultivation on penicillin-free media. The rate of mutation to sensitivity varied among her strains and could not be detected at all in some.

Voureka (81) has recorded the interesting observation that some strains of penicillin-resistant staphylococci and streptococci lost their resistance during exposure to a penicillin-sensitive microorganism or to autolysates and even to extracts of sensitive bacterial cells. She first demonstrated this phenomenon by growing a resistant strain in mixed culture with a sensitive strain of another species from which it could be readily separated. Although two workers (82, 83) have been unable to repeat Voureka's observation, it has been confirmed by Winner (84) and by George & Pandalai (85). The latter workers found the sensitizing principle to be present in the ribonucleic acid fraction isolated from sensitive microorganisms.

*Morphology of resistant cells and colonies.*—Many, but not all, investigators have noted changes in the morphology of bacteria which had acquired resistance to penicillin. The most striking alteration was increased size and atypical shape of resistant cells growing on penicillin media and increased or decreased affinity for the customary stains (57, 58, 63, 64, 71, 72).

A few workers found differences in colonial morphology between resistant and sensitive parent strains. Schnitzer *et al.* (86) noted that the resistance of a strain of *Micrococcus pyogenes* var. *albus* to penicillin was associated with the formation of small colony variants (G forms). They also found that exposure of the same strain to barium chloride resulted in the production of G forms which possessed some degree of resistance to penicillin. They concluded that the resistance of small colony variants to penicillin could not be considered a specific drug resistance.

Eriksen (60) also noted the association of the appearance of small colony variants in a culture of *M. pyogenes* var. *aureus* with the rise in resistance to penicillin. The slow growing small colony strains were found to be unstable and were no longer present after the loss of resistance during subcultivation on penicillin-free media. One strain remained stable after two months' subculture on penicillin-containing media and was found to be stimulated by noninhibitory concentrations of the drug. Enhancement of growth in the presence of penicillin was also noted by Miller & Bohnhoff in two strains of highly resistant meningococci (79).

Resistant strains of *M. pyogenes* var. *aureus* lost their ability to produce their characteristic pigmentation (57, 63, 64), and strains of streptococci lost

their hemolytic activity (65) or underwent transient changes from beta to alpha or gamma hemolysis while growing on maximum concentrations of penicillin (67, 68, 69).

*Biochemical and immunological characteristics.*—The *Annual Review of Microbiology* has already published exhaustive discussions of the literature on the various biochemical changes in bacterial cells resulting from the action of penicillin (87, 88, 89). Only those studies which concern the changes occurring during the development of penicillin resistance are discussed here.

Certain alterations in the biological processes of bacteria as they acquired resistance to penicillin have been observed by almost all investigators of this phenomenon. The first to be described were those which can be easily detected by the ordinary techniques of bacteriology; viz, slower rate of reproduction, slower rate of fermentation of the sugars characteristically attacked by the strain, and reduction in virulence for experimental animals. In most instances, however, virulence could be restored by animal passage.

The resistant strain of *M. pyogenes* var. *aureus* studied by the team of investigators at Oxford (55) was found to produce all its characteristic biochemical reactions including hemolysis, albeit more slowly than its sensitive parent strain. This fact was attributed to the slower growth rate of the resistant strain. Bellamy & Klimek (90) found that a strain of *M. pyogenes* var. *aureus* lost its ability to grow anaerobically after it had acquired resistance to 1 mg. of penicillin per ml. They suggested that loss of anaerobiosis was the cause for the slower growth rate of penicillin-resistant organisms and postulated that organisms unable to grow aerobically would develop little resistance to penicillin. They thought this might explain the facility of staphylococci to develop resistance as compared with streptococci or pneumococci. Their strain of staphylococci (64) became a gram negative coccobacillus and lost its characteristic biochemical reactions including hemolytic activity. These characteristics returned to normal when the strain lost its resistance after cultivation in penicillin-free media. However, repeated transfer in high concentrations of penicillin produced a stable strain which retained its resistance in drug-free media, which was unable to ferment the usual sugars, to grow in 6.5 per cent sodium chloride, or to reduce nitrates, and which no longer required nicotinic acid (78).

In a detailed study of the amino acid metabolism of the staphylococci Gale (91) found the assimilation of glutamic acid to be inhibited by penicillin. As strains acquired resistance during subcultivation in increasing concentrations of penicillin, they lost much of their ability to assimilate glutamic acid but were able to synthesize their necessary amino acids and eventually required only ammonia and glucose. Two strains of staphylococci which were trained to dispense with all amino acids except cystine and histidine were found to be more resistant to penicillin than their parent strains (92). In other words, mutants which were selected for their ability to synthesize all but these two amino acids were not inhibited by penicillin. Reverse mutants which lost ability to synthesize amino acids regained sensitivity to penicillin.

It is clear, therefore, that the development of resistance in staphylococci

by the customary method of cultivation in subinhibitory concentrations of penicillin resulted in the selection of mutants which had acquired the capacity to synthesize their essential amino acids and were, therefore, independent of the interference by penicillin with the assimilation of amino acids necessary for growth (93). Another instance of the relation of synthetic ability to penicillin resistance is supplied by Plough & Grimm (94) who found that a mutant obtained from *Salmonella typhimurium* which required cysteine was much more sensitive to penicillin than the parent strain.

In a systematic study of the changes in enzymatic activity of Groups A and C beta hemolytic streptococci which accompanied the development of penicillin resistance *in vitro*, Gezon & Fasan (95) found that the production of streptolysin S and streptokinase was reduced. In some strains of Group A streptococci, there was also reduced production of proteinase.

The type specificity of pneumococci and meningococci was retained unchanged as strains of these organisms acquired resistance. In the case of the meningococcus (72), careful examination by a variety of immunological methods failed to detect any antigenic differences between the sensitive and resistant strains. On the other hand, loss of group specificity was observed in about half of the strains of hemolytic streptococci, Groups A, B, and C, studied by Gezon (67, 68, 69).

*Penicillinase production.*—The ability to produce penicillinase is a characteristic which differentiates natural from acquired resistance. Most naturally resistant strains of *M. pyogenes* var. *aureus* have been found to produce penicillinase, whereas most strains which have acquired resistance *in vitro* do not (55). A notable exception is the gram negative strain of Bellamy & Klimek (78) which produced penicillinase, but only when growing in the presence of penicillin.

*Experimental studies in vivo.*—The development of some degree of penicillin resistance in two strains of the pneumococcus by repeated passage through mice treated with penicillin was reported by Schmidt & Sesler (96). This was manifested by a decrease in the percentage of survival after treatment with standard doses of penicillin. At the end of the experiments, *in vitro* sensitivity determinations showed a four-fold increase in resistance of both strains. Miller & Bohnhoff (97) raised the $PD_{50}$ (dose of penicillin which protected 50 per cent of the mice) of a strain of meningococcus from 10 units to 1,700 units during 61 passages through mice treated with subcurative doses of the drug. Sensitivity determinations showed comparable increases in resistance.

Gezon & Collins (98) were unable to increase the resistance of two strains of Group A hemolytic streptococci during repeated passage through mice or in embryonated eggs treated with penicillin. Two strains each of Group B and Group C streptococci, however, showed a 30 to 40-fold increase during passage in embryonated eggs, but this was not associated with a corresponding rise in penicillin resistance as determined *in vitro*. Passage of these Group B and C strains in mice caused only a questionable increase in resistance *in vivo*. Rake *et al.* (58) were unable to demonstrate a rise in penicillin re-

sistance in a strain of *M. pyogenes* var. *aureus* during serial passage through treated mice. An apparent increase was attributed to enhancement of virulence.

*Clinical observations.*—The development of penicillin resistance in patients during the course of treatment has been described in a number of clinical reports. These observations have been made in cases in which therapeutic failure has led to determination of the sensitivity of the infecting microorganism and comparison with its sensitivity before treatment. Some of these reports are pertinent to the present discussion, particularly when they deal with infections not exposed to secondary invasion with penicillin-resistant contaminants.

The reports of infections in sites exposed to contamination such as burns, open wounds, draining osteomyelitis cavities, etc. are open to question. When a penicillin-sensitive microorganism is recovered from such a lesion before treatment and a penicillin-resistant one during or after treatment, this change may be explained by any of the following assumptions: (*a*) that the strain has actually acquired resistance *in vivo* as the result of penicillin therapy, (*b*) that the sensitive strain has been eliminated by treatment but replaced by a more resistant strain of the same bacterial species; i.e., the site has become secondarily infected by a resistant contaminant of the same species, an occurrence which is easily possible in the case of staphylococci (99), or (*c*) that the bacterial population causing the infection consisted of a mixture of many sensitive and a few resistant individuals which were not detected in the original culture because they were overgrown by the more numerous penicillin-sensitive cells. It should be pointed out, however, that the rate at which resistance developed in a number of such instances (56, 100, 101, 102) did not exceed the increase observed in infections occurring within the blood vascular system or in closed cavities not subject to contamination.

A number of cases of subacute bacterial endocarditis have been reported in which penicillin resistance has been shown to have developed during the course of treatment. Most of them have been caused by alpha hemolytic streptococci. Typical examples are: a 16-fold increase in 8 days (103), an 8-fold increase in 16 days (100), a 10-fold increase in 44 days (104), a 30-fold increase in 6 months (105), but also a 2,084-fold increase in 31 days (103). The development of resistance in other bacterial species causing septicemia has also been reported (61, 105, 106, 107).

Increase in penicillin resistance has been observed in a sufficient number of infections which could not have become contaminated to establish the point that bacteria can acquire resistance in man during the course of treatment. This is not surprising since increase in resistance has been demonstrated experimentally *in vitro* and *in vivo.* The relative number of instances, however, in which penicillin resistance was found to develop is insignificant in comparison with the number in which treatment has been successful. These exceptional instances are due either to inadequate treatment or to the location of infection in areas to which penicillin is not readily transported

by the blood supply. The development of penicillin resistance during the course of most infections with sensitive organisms is rarely a serious clinical problem because of the highly effective antibacterial properties of this agent and its low toxicity, which permits the administration of doses larger than are actually necessary to combat infection.

*Origin of resistance.*—A satisfactory explanation of the development of bacterial resistance to sulfonamides was furnished by the work of Oakburg & Luria (51) and of resistance to penicillin by Demerec (108), Luria (109), and Klein (110). Increase in resistance during cultivation in subinhibitory concentrations of the drug results, according to Demerec's theory, from the appearance in the culture of resistant variants. These variants arise spontaneously in the bacterial population by the process of random mutation. The drug does not induce the mutation, but acts merely as a selective agent which permits the multiplication of the resistant variant and suppresses the growth of all sensitive members of the population.

Demerec postulates that the bacterial cell contains a number of genes concerned with resistance and that they are of equal "potency." (The case of streptomycin resistance is exceptional and will be discussed below.) A mutation in any one of these genes endows that cell and its progeny with the ability to multiply in the presence of a concentration of the drug somewhat greater than that tolerated by its parent cell. As successive mutations occur the level of resistance gradually rises in a step-wise fashion.

The differences in the ability of various bacterial species and of strains within each species to acquire resistance is explained on the basis of differences in their inherent rates of mutation. Miller & Bohnhoff found resistance to develop most rapidly *in vitro* when vigorous growth was maintained in the strain as it was undergoing serial transfer (71). In the case of the gonococcus, this was accomplished by alternating penicillin-containing and penicillin-free media (72). This procedure provided larger numbers of bacteria from which resistant mutants could be selected on penicillin media.

The validity of the mutation theory of the development of resistance has been questioned by Eriksen (111), who believes that penicillin-resistant mutants do not arise spontaneously but only as a result of exposure to penicillin. Space does not permit adequate discussion of his argument and the experimental data supporting it. Hinshelwood's treatment (112) of adaptation of bacterial cells to the action of drugs is concerned with the kinetics of the biochemical processes involved rather than with the genetic changes.

## Aureomycin

Only a few papers have thus far appeared dealing with the development of bacterial resistance to aueromycin. Enough observations seem to have been reported, however, to demonstrate that resistance can develop in some strains of bacteria during repeated cultivation on aureomycin-containing media. When it does occur *in vitro*, it follows the pattern of the sulfonamides and penicillin; i.e., it rises slowly and in a step-wise fashion.

Aureomycin is not very stable at 37°C. and at the pH of ordinary bacteriological media. Demerec (113) has pointed out that the gradual loss of potency which takes place under these conditions adds to the difficulty in making precise determinations of resistance. Colonies which appear after two or three days' incubation may be either slow growing resistant organisms, or sensitive ones which were able to multiply only after the effectiveness of the drug has diminished below the inhibitory level.

*Experimental studies.*—Finland *et al.* (114) found a 32-fold increase in a strain of *Aerobacter aerogenes* during 30 transfers in aureomycin-containing media, but no further increase during 40 additional transfers. Price *et al.* (115) studied the development of resistance n a variety of bacterial species by serial transfer in broth containing the drug. After 14 subcultivations, the maximum increase was 533-fold in a strain of *Proteus vulgaris* and the minimum increase was 2-fold in a strain of *Sarcina lutea;* one strain of *M. pyogenes* var. *aureus* did not acquire resistance after 14 transfers in aureomycin-containing broth.

In a study of the incidence of aureomycin-resistant mutants of four strains of *M. pyogenes* var. *aureus*, Lankford & Lacy (116) found that one strain developed very small colonies. Growth of organisms from these colonies was stimulated by aureomycin although the drug was not an absolute growth requirement.

Gezon & Fasan (117) studied the rate of development of resistance by 23 strains of Groups A, B and C hemolytic streptococci during 40 serial transfers on aureomycin-containing agar. They found a maximum increase of 60-fold in a Group C strain and a minimum of only 2-fold in a Group A strain. Resistance was not maintained during serial transfer on control media or passage through normal mice. Mouse virulence was reduced in the organisms which had acquired the highest degree of resistance.

Some of their strains, while growing on maximum tolerated concentrations of aureomycin, showed temporary changes in hemolytic activity; i.e., from beta to alpha, or complete loss. Some produced less streptokinase and showed less ribonuclease activity than the sensitive strains from which they were derived. There was no change in streptolysin S production or proteinase activity. Resistance failed to develop *in vivo* in one Group A strain during repeated passage in aureomycin treated mice and embryonated eggs.

*Clinical studies.*—In a report on the treatment of four patients with subacute bacterial endocarditis, Harvey *et al.* (118) noted that in two of the patients, the causative organism (one a *Streptococcus fecalis*, and one an alpha *Streptococcus*) increased 8 and 16-fold respectively during 10 weeks' treatment. In a large series of patients treated with aureomycin for a variety of infections, Finland *et al.* (119) reported a 4-fold increase in resistance of *A. aerogenes* and a 33-fold increase in *Salmonella typhosa*.

## Chloramphenicol (Chloromycetin)

The development of resistance to chloramphenicol by repeated subcultivation was observed by McLean *et al.*(120) in a number of gram nega-

tive bacilli during 7 to 12 transfers in broth containing increasing concentrations of the drug. The increase varied from 2 to 50-fold. There was no increase in the resistance of *Rickettsia prowazeki* during 13 transfers in chick embryos treated with the drug.

Alexander *et al.* (121) noted that a few colonies of *E. coli* and *Salmonella*, including *S. typhosa*, grew out on concentrations of 10 μg. per ml. but none of them grew in subcultures on agar containing this same concentration of chloramphenicol. Yow & Spink (122) found no evidence of acquired resistance in five strains of *Brucella* during 17 transfers in media containing the drug. Resistance to chloramphenicol follows the pattern observed with the sulfonamides, penicillin, and aureomycin.

## Streptomycin

The development of resistance to streptomycin is a more complicated phenomenon than the development of resistance to the antibacterial agents already discussed. Although it may occur gradually as it does to the others, resistance to streptomycin differs in two important respects: (*a*) it may also increase sharply because of the emergence of mutants which are completely fast to streptomycin; (*b*) a second type of resistant mutant may appear which is dependent upon streptomycin for growth. For the sake of convenience, the development of resistance and dependence will be discussed separately.

*Development of resistant mutants in vitro.*—Miller & Bohnhoff (123) observed that meningococci and gonococci could acquire resistance to streptomycin much more rapidly than to penicillin during surface cultivation on agar containing the drug. After two or three transfers, abundant growth occurred on media containing 50,000 μg. per ml.

Murray *et al.* (124) found that serial cultivation of sensitive strains of gram negative bacilli in broth and on agar containing streptomycin resulted in a step-wise development of resistance not unlike that observed with penicillin. Sooner or later, however, there was an abrupt rise to very high levels of resistance, and this was found to occur much earlier in the series when transfers were made with very large inocula onto surface cultures.

The explanation for this sudden increase in resistance was supplied by the observation that sensitive strains contained small numbers of mutants (roughly 1 in $10^9$) which were highly resistant to streptomycin. This finding was demonstrated independently in meningococci (125, 126), *Hemophilus influenzae* (127), *Shigellae* (128), *E. coli*, *Proteus*, and *M. pyogenes* var. *aureus* (110) and later for most other bacterial species (116, 122, 129 to 132) including beta hemolytic streptococci (133). Although such mutants were not described in some of the early reports, they undoubtedly accounted for the sudden rise in resistance which was observed (63, 123, 134, 135, 136).

In general, the highly resistant strains were much more stable than those which had gradually acquired low degrees of resistance. These latter tended to lose their resistance in the step-wise fashion in which it was acquired (128). This difference in stability may well be due to the technical difficul-

ties encountered in detecting back-mutation in highly resistant strains since many of these grow at the same rate as their sensitive parent strains.

*Changes in macroscopic and microscopic appearance of resistant strains.*—A distinct yellowish pigmentation was observed in the colonies of highly resistant variants of the meningococcus (125, 126) and loss of pigment formation in some strains of *Pseudomonas* (137) and *M. pyogenes* var. *aureus* (63). The hemolytic activity of beta streptococci showed transient changes from beta to alpha or loss of alpha (138). Microscopically, the cells of some resistant strains have shown minor abnormalities in size and shape (124, 139, 140).

*Biochemical characteristics of resistant strains.*—Reduction in the rate of growth has been reported for the resistant variants of *M. pyogenes* var. *aureus* (63) and *Mycobacterium tuberculosis* (141). Some resistant strains of *Shigella* showed atypical reactions in Russell's double sugar agar (128), some strains of *Salmonella* lost their ability to produce gas, and one of these did not produce hydrogen peroxide (137). Other significant changes in carbohydrate metabolism have been noted in *E. coli* (142).

Resistant strains of several species of nonpathogenic mycobacteria were found to require more streptomycin to inhibit their oxidation of benzoic acid (143). Most strains of hemolytic streptococci, Groups A, B, and C, produced less streptokinase and streptolysin S (138) and the proteinase and/or ribonuclease activity of some was also reduced (95).

*Experimental studies in vivo.*—Highly resistant meningococci were found to be as virulent for mice as the parent strains from which they were derived. On the other hand, all of seven strains of resistant *Salmonella* (137) and seven of eight strains of beta hemolytic streptococci (138) lost virulence for mice.

Strains of *M. tuberculosis* which had acquired resistance *in vitro* and resistant strains isolated from streptomycin-treated patients were found to be fully virulent for experimental animals and produced infections which could not be controlled by streptomycin (144 to 147). The development of resistant strains of tubercle bacilli *in vivo*, i.e., in animals during prolonged treatment with streptomycin, was first demonstrated by Feldman *et al.* (148). Recently, Youmans *et al.* (149) have shown that more highly resistant strains were recovered from infected mice treated with large doses of streptomycin than from those treated with small doses.

*Clinical observations on the development of resistance.*—The appearance of resistant strains during streptomycin treatment in patients was first observed in infections of the urinary tract with the common gram negative bacilli (124, 150, 151). The development of resistance during the course of treatment was also observed in meningitis caused by *H. influenzae* (127, 152 to 156), *Pseudomonas* and *E. coli* (153), and *Achromobacter* (157), in endocarditis caused by *Brucella* (158), *Streptococcus* (159, 160), and an unclassified gram negative bacillus (159) as well as in a variety of infections caused by a number of other bacterial species (161, 162, 163).

In fact it is generally recognized in clinical practice that infections with bacteria other than the slow growing acid-fast bacilli must be brought under

control within 72 to 96 hr. if streptomycin is to be effective at all. The same rule applies to the administration of streptomycin by mouth to reduce the bacterial flora of the lower gastrointestinal tract in preparation for major operations on the colon. The bacterial population falls rapidly during the first two days, but after the third day, rises suddenly and consists largely of streptomycin-resistant microorganisms (164, 165, 166).

Because of its slower rate of reproduction, the tubercle bacillus acquires resistance much more slowly than the bacteria mentioned above. For this reason, tuberculous infections respond to streptomycin therapy for a much longer time than do those caused by more rapidly growing bacteria. Eventually, however, resistant strains usually appear. After this has happened, the administration of the drug is no longer efficacious. Resistance thus acquired is a permanent characteristic and may be detected for a long time after termination of treatment (167, 168, 169). For a more complete discussion of the factors involved in the development of resistance by tubercle bacilli, the reader is referred to the reviews by Steenken & Wolinsky (170) and by D'Esopo (170).

The importance of the clinical problem resulting from the development of streptomycin resistance has led to various attempts to delay its occurrence. At present, the most promising is the addition of some other tuberculostatic drug such as *p*-aminosalicylic acid (PAS). Tubercle bacilli have been found to develop resistance to streptomycin much more slowly *in vitro* and *in vivo* when the drug is combined with PAS (171 to 176).

*Development of dependent mutants in vitro.*—Streptomycin-dependent mutants as well as resistant mutants were observed by Miller & Bohnhoff (125, 126) when sensitive strains of the meningococcus were exposed to high concentrations of the drug. They have been isolated from a sufficient variety of bacteria, including both gram negative and gram positive species, to indicate that their occurrence represents a common phenomenon among microorganisms (122, 177 to 183).

Dependence on streptomycin was demonstrated *in vivo* by inoculating mice with dependent strains of meningococci or *E. coli* (126, 170). No infection occurred unless the animals were treated with the drug; but infection in the treated animals developed into a fatal septicemia. Organisms recovered from the hearts' blood of the latter group were shown to be dependent *in vitro* upon streptomycin for growth.

*Morphology of dependent colonies and cells.*—Macroscopic differences in size, structure, and pigmentation of dependent colonies were described for some dependent strains (125, 126, 178). Streptomycin-dependent organisms grown on adequate concentrations showed no abnormalities and only minor ones when grown on suboptimal concentrations (126, 180). However, dependent strains of *E. coli* inoculated into streptomycin-free broth were found to increase enormously in length, as if the drug acted as a factor necessary for cell division rather than for building cellular protoplasm (184).

*Growth characteristics and biochemical changes.*—Although streptomycin-dependent strains are able to multiply within very wide limits of concentra-

tion, most of them grow best within a range which seems high for an essential growth factor, e.g., 0.1 to 0.4 mg. per ml. for meningococci. Stability of these dependent mutants varied from strain to strain. Mutation may occur either to sensitivity or to ordinary resistance (178, 185). Abnormalities of biological behavior include slower rate of growth and occasional deficiency in carbohydrate metabolism (142, 180, 186).

*Substitutes for streptomycin as a growth requirement.*—Winsten (187) found that a dependent strain of *M. pyogenes* var. *aureus* was able to utilize N-*n*-propyl-, N-*n*-butyl-, and N-*n*-hexylstreptomycylamine in place of streptomycin. Rake (188) reported some activity in two preparations of inositol and also in streptobiosamine and N-acetyl streptomycin A, but the activity of these latter two might have been explained by contamination with streptomycin.

*Demonstration of dependent mutants in vivo.*—Miller & Bohnhoff (189) isolated streptomycin-dependent microorganisms from the throats of patients undergoing treatment with the drug and also from the upper respiratory and lower intestinal tract of mice and rabbits after 7 to 10 days' treatment with streptomycin. Lenert & Hobby (190) isolated dependent strains of tubercle bacilli from streptomycin treated mice, but infection with these strains was not promoted by streptomycin treatment. Spendlove *et al.* (191) isolated two strains of *M. tuberculosis* from a patient during and after streptomycin therapy which grew more abundantly in streptomycin-containing media. Other variants of tubercle bacilli, *H. influenzae* and *Brucella* have been found *in vitro* which were not strictly dependent on streptomycin but which grew more abundantly in its presence (127, 158, 182).

*The origin of streptomycin resistance and dependence.*—Consideration has already been given to the theory of Demerec and Oakburg & Luria which is generally accepted in explanation of the development of resistance to the sulfonamides and penicillin. That discussion must be supplemented in the case of streptomycin for resistance to streptomycin may increase either slowly by small increments, or it may rise abruptly to a very high level. Demerec explains this difference by assuming that the genes responsible for streptomycin resistance are of unequal "potency." Mutation in a gene of low potency results in a slight increase in resistance. All the genes involved in sulfonamide and penicillin resistance are assumed to be of low potency, an explanation which accounts for the fact that resistance to these drugs never rises abruptly to a high level.

Linz (192) has raised some interesting objections to the theory that streptomycin resistance results from spontaneous mutation. He believes that it is induced by the direct action of the drug on the bacterial cells. Streptomycin dependence like streptomycin resistance seems to be most easily explained in accordance with current genetic theory as resulting from the appearance of dependent variants by the process of random mutation which is constantly taking place in the population of a susceptible strain. On ordinary media, such mutants are of necessity nonviable because they occur in the absence of streptomycin.

The most helpful clue to the nature of the change accompanying streptomycin-dependent mutation is supplied by the observation of Paine & Finland (179, 180) that the minimum concentration of streptomycin required by a dependent mutant is about the same as that which completely inhibits the growth of its susceptible parent strain. The mutation presumably involves a vital process which must undergo a relatively simple change. The process can be most easily explained by assuming streptomycin to be an analog of a co-enzyme in some reaction essential for growth. In susceptible cells, this reaction is blocked by streptomycin, but as a result of the mutation, some minor change occurs which permits the drug to function as the co-enzyme and thereafter to be essential for growth.

## Summary

This review has been limited perforce to the chemotherapeutic agents most widely used today; viz, the sulfonamides, penicillin, aureomycin, chloramphenicol, and streptomycin. Resistance can be acquired by most, if not all, species of bacteria, but more rapidly by some than others. It can be demonstrated experimentally *in vitro* and *in vivo* and also in man during the course of treatment.

With the exception of streptomycin, resistance to these drugs always develops slowly and in a step-wise fashion. In the case of streptomycin, it may also increase suddenly to complete fastness and may be accompanied by the emergence of strains which are dependent upon streptomycin for growth. Acquired resistance is generally believed to result from the appearance of resistant individuals in the bacterial population by the process of spontaneous mutation. According to this theory, the presence of the drug merely prevents the survival of all but the resistant mutants.

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# CHEMOTHERAPY OF VIRUS AND RICKETTSIAL INFECTIONS[1]

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In this review, it is our intention to discuss chemotherapy not only as treatment of infectious disease but also from the more fundamental aspect of the biochemistry of virus-host cell relationships. Knowledge of what substances and experimental procedures inhibit the growth of viruses contributes not only to chemotherapy but also may lead to better understanding of the mechanisms of cell invasion and the relation of enzyme systems of the host cell to growth of viruses.

Significant differences in the biochemical nature of various species of viruses are becoming apparent. At the top of the scale are the rickettsiae and some of the larger viruses which, from chemotherapeutic as well as other observations, seem to resemble bacteria. The growth of intermediate viruses such as influenza and mumps has been inhibited in the laboratory by chemical procedures quite different from those successful with the larger agents. None of these methods has yet proved effective against the smaller viruses such as poliomyelitis. Chemotherapeutic studies indicate that biochemical similarities may parallel evolutionary relationships among viruses, but they have also disclosed among the rickettsiae and larger viruses wide variations in sensitivity to a given chemotherapeutic agent.

## Chemotherapy of Rickettsial Infections

*Antibiotics.*—Penicillin and streptomycin are less active *in vivo* against rickettsiae than bacteria, while chloramphenicol (chloromycetin) and aureomycin are each almost equally effective against bacterial and rickettsial infections.

Penicillin in large doses was first found to inhibit the growth of the agent of murine or endemic typhus, *Rickettsia mooseri*, in chick embryos (1, 2, 3) and in the dba strain of mice (4). Greiff & Pinkerton (4) observed that certain of the crystalline penicillin products, especially penicillin X, were much more active than others, and some commercial samples of penicillin obtained during the years of 1945 through 1947 showed no activity against murine typhus in chick embryos. Only a few instances of the use of this antibiotic in the treatment of human rickettsial infections are recorded and the results in epidemic typhus (5) and scrub typhus (6) were negative or inconclusive. Penicillin is apparently ineffective against experimental Rocky Mountain spotted fever in the guinea pig (7).

Streptomycin also shows some rickettsiastatic activity against experimental infections in chick embryos with murine typhus, epidemic typhus,

[1] This review covers approximately the period from January, 1945 to March, 1950.

Rocky Mountain spotted fever, Q fever, rickettsial pox, and a vole *Rickettsia*, but not against scrub typhus (8, 9, 11). Very large doses of 10 to 20 mg. appeared about equal in effect to *p*-aminobenzoic acid (PAB) (9). Synergistic action of streptomycin and PAB was noted. This antibiotic also modifies the infection produced by the rickettsiae of Q fever in the guinea pig (10).

Chloramphenicol has been obtained as a synthetic product with chemotherapeutic activity equal to that of the natural substance (13). Efficacy has been demonstrated against a wide variety of rickettsial infections including endemic and epidemic typhus, scrub typhus, Rocky Mountain spotted fever, Q fever, and rickettsial pox in the chick embryo (12 to 15), endemic typhus, scrub typhus, and rickettsial pox in mice, (12, 15), and scrub, epidemic and endemic typhus in man (16 to 21). The results with Rocky Mountain spotted fever in guinea pigs were, however, quite irregular, and a considerable portion of the treated animals died (15). This was attributed to low blood levels of chloramphenicol in this species. The results of treatment of this disease in man (22) are reported good although no concurrent untreated cases are available for comparison. In tissues of mice infected with *R. tsutsugamushi*, the organisms could be recovered by animal passage even after 100 days of treatment with chloramphenicol, but in some of the mice infected with rickettsial pox, sterilization of the infection was demonstrated (15). At a concentration of 1.25 mg. per ml., chloramphenicol failed to display direct rickettsiacidal action *in vitro* against *R. tsutsugamushi*. Smith (14) reported that chloramphenicol on a weight basis was many times as effective as streptomycin, methylene blue, or PAB against epidemic typhus in the chick embryo, and there is no doubt that the therapeutic index of chloramphenicol is much more favorable than that of the latter two substances.

The results with chloramphenicol in the treatment and prophylaxis of scrub typhus (tsutsugamushi disease) in human beings were impressive (16, 17, 18). Treatment of 22 cases of epidemic typhus resulted in recovery and defervescence within three days, while among 50 untreated cases of typhus occurring in the same epidemic, the mortality was 28 per cent (19, 20).

Aureomycin apparently has antirickettsial activity at least as great as that of chloramphenicol although significant *in vitro* activity against rickettsiae is not demonstrable. Experimental infections with many species of rickettsiae were reported by Wong & Cox (23) to be favorably influenced by aureomycin. In mice infected intravenously with murine typhus, the very small dose of 0.01 mg. of aureomycin per day produced some effect. Therapeutic effects with 1 mg. per day were demonstrable when treatment was delayed for as long as 96 hr. or at a time when the mice were critically ill with murine typhus infection. Mice treated with 1 mg. of aureomycin survived intraperitoneal inoculation with 1,000,000 lethal doses of scrub typhus. In guinea pigs infected with Rocky Mountain spotted fever, epidemic typhus, or Q fever (23, 24), aureomycin suppressed the febrile reac-

tions and other signs of illness when given 1 to 2 days after inoculation. In some guinea pigs receiving small doses of rickettsiae and treated before symptoms appeared, no latent infection could be found by subinoculation indicating a rickettsiacidal action *in vivo*. Q fever was somewhat less responsive to doses of aureomycin of 1 mg. or under.

A number of investigators have reported favorable results with aureomycin in the treatment of human infection with Rocky Mountain spotted fever (26 to 30). The results were superior to those obtained with PAB (28) in that a greater reduction of the period of acute symptoms and fever was obtained, and response appeared to be uniformly good despite delay in the treatment of some cases. In patients with typhus fever, presumably of the murine variety, dramatic improvement and prompt clinical remission occurred within 24 to 48 hr. after the start of treatment (31).

The response of Q fever in human beings (25) to aureomycin appears to have been somewhat less prompt than that of other rickettsial infections. In some cases, relapses occurred following cessation of therapy, but the patients usually became afebrile after receiving additional aureomycin. One chronic case of Q fever failed to respond even to large doses of aureomycin. Reports on the action of other antibiotics on rickettsiae are not available except for bacitracin (32) and tyrothricin (36) which were found to be ineffective.[3]

*p-Aminobenzoic acid and sulfonamides.*—Sulfadiazine and other sulfonamides have been tested in experimental infections with Rocky Mountain spotted fever and typhus in guinea pigs (7, 33, 34) and against murine typhus in chick embryos (1, 37) and mice (5, 36) with negative results. In fact, some investigators believe that these drugs increased the severity of the infection (5, 33). Negative results have also been reported in human rickettsial infections (36).

An observation of considerable theoretical interest was the demonstration of activity against experimental typhus in mice of a class of related compounds, the sulfonamidobenzamidines (35, 36). Under the experimental conditions in mice, these substances showed greater activity than PAB, but they were ineffective in guinea pigs and human beings (36) possibly because a relatively greater toxicity in the latter hosts limited the dosage. Unlike sulfonamides, their activity was not reversed by PAB.

The therapeutic effect of PAB on experimental typhus was first observed by Snyder, Maier & Anderson in 1942, but their results were not published until 1944 when favorable effects in the human disease were also described (5). The rickettsiastatic activity extended to several species of rickettsiae in the chick embryo (37, 38), Q fever and scrub typhus being least affected. Contrary to the results in chick embryos, PAB was found to be highly effective against scrub typhus in gerbilles (39, 40, 41). PAB lowered the mor-

[3] Since this review was completed, terramycin, a new antibiotic with antirickettsial properties similar to aureomycin and chloramphenicol has been described. Papers on terramycin by several groups of investigators will appear in the Annals of the New York Academy of Sciences, 1950.

tality from murine typhus in mice (2) and diminished the febrile reaction to Rocky Mountain spotted fever in guinea pigs (42). It was found that other isomers and related compounds were inactive, which suggested a high degree of specificity and possible action on an enzyme system.

Greiff & Pinkerton (43) were able to demonstrate that PAB increased the oxygen consumption of both normal and infected chick embryos, whereas the meta and ortho compounds which were not rickettsiastatic had no effect. This finding appeared to be a counterpart of the observation that sublethal amounts of potassium cyanide which decreased metabolism and oxygen consumption favored the growth of rickettsiae under certain conditions. Incubation of infected chick embryos at 40°C. inhibited growth of rickettsiae; this effect of elevated temperature was neutralized by potassium cyanide indicating a possible natural rickettsiastatic effect of cyanide sensitive enzyme systems in the entodermal cells of the yolk sac (44). Since potassium cyanide failed to effect the rickettsiastatic activity of either PAB or thionine dyes, Greiff & Pinkerton concluded that the action of these agents was on an enzyme system different from that concerned in growth inhibition at elevated temperature. Pteroylglutamic acid had no effect either on the growth of the rickettsiae in chick embryos or on the oxygen consumption.

PAB, when given in the first week of illness in doses sufficient to produce blood levels over 10 mg. per cent, has produced significant therapeutic effects in human infection with epidemic typhus (5, 45) and murine typhus (46). Therapeutic value in scrub typhus has also been ascribed to PAB, but larger doses are apparently required (45, 47). A number of investigators (48 to 54) have reported favorable results in Rocky Mountain spotted fever. The drug produces various toxic manifestations in man.

*Thionine dyes.*—The effect of thionine dyes on rickettsial infection is of interest because of the action of these substances as accessory hydrogen acceptors in certain metabolic systems. Peterson (55) first observed that toluidine blue was quite effective in preventing deaths from murine typhus in mice. These observations were later extended to other species of rickettsiae and other thionine dyes including methylene blue in cotton rats, mice, and chick embryos (44, 56 to 59). The results in man were disappointing (60).

It has been suggested (61) that the mode of action of thionine dyes is independent of the *in vitro* rickettsiacidal activity and that it might involve their action as respiratory catalysts in preventing cell damage from rickettsial multiplication or toxins. In mice, multiplication of rickettsiae continues in certain organs despite therapy with methylene blue. Increased oxygen tension is said to augment the therapeutic activity of methylene blue (59). The antirickettsial effect was limited to phenothiazines containing methyl- or ethyl-amino groups at the 3 and 9 positions. Phenazines such as neutral red and structurally similar compounds were tested and found to be inactive.

*Acridines.*—This class of substances is of continuing interest because of

reports of chemotherapeutic activity not only against rickettsiae but also against viruses of the psittacosis-lymphogranuloma group, bacteriophage, and influenza. Nitroakridin 3582 [3-nitro,6,7,-dimethoxy-9-(2-hydroxy-3-diethyl-amino-propyl-amino)-acridine] and its arsenical salt, rutenol, appear to be the most active substances in this class against rickettsiae of several species (62). In addition 3-nitro-9-amino acridine was found to be more effective but also more toxic than Nitroakridin 3582 against *R. mooseri* in chick embryos while acriflavine displayed some antirickettsial activity when used near the toxic level but was apparently inactive in mice (58). Proflavine, atabrine, and other acridines were inactive against murine typhus in mice and chick embryos (32, 58, 62). Large amounts of yeast nucleic acid apparently blocked the antirickettsial action of Nitroakridin 3582. Reports by German workers on the clinical use of these compounds are inconclusive (63, 64), and their high toxicity is a contraindication. None of the antimalarial quinolines or related compounds has shown activity against rickettsiae (32).

*Nitrocompounds.*—In addition to chloroamphenicol, which contains a nitrobenzene nucleus, and Nitroakridin 3582, several other nitrocompounds have been tested against murine typhus. Apparently conflicting results are reported for 1,1,1,trichloro-2,2,bis-(*p*-nitrophenyl)-ethane, and for *p*-nitrobenzoic acid and some of its derivatives (32, 65, 66). 2,4,Dinitrophenol, a substance which causes marked stimulation of oxygen uptake by tissues *in vitro*, had no affect on the growth of rickettsiae (44).

*Vitamin deficiency.*—Experimental evidence that several forms of vitamin deficiency increase the susceptibility of animals to rickettsial infection (68 to 71) has been reviewed recently (67).

*Other substances.*—Salicylic acid and malachite green are reported by two independent groups of investigators to increase the survival time in experimental murine typhus of mice (66) and chick embryos (32). Slight protective effects of ascorbic acid are reported against murine typhus infections in chick embryos (32) and epidemic typhus in guinea pigs (71). Several other classes of substances have also been reported to have antirickettsial properties (32).

## The Psittacosis-Lymphogranuloma Venereum Group (Chlamydozoaceae)

Drugs and antibiotics that have been found to inhibit the Chlamydozoaceae are usually also effective chemotherapeutically against bacterial infections indicating a close biochemical relationship to bacteria. These agents differ from rickettsiae in that some of them are inhibited by sulfonamides while none are affected by PAB (38, 72, 76), thionine dyes (73, 74), and streptomycin (75, 76). Penicillin is more active against Chlamydozoaceae than rickettsiae, but the opposite is true of chloramphenicol.

*Antibiotics.*—Penicillin is inhibitory to all of the viruses of the psittacosis-lymphogranuloma group (75 to 82) in mice infected by the respiratory, intraperitoneal, or intravenous routes and is also virustatic in chick

embryos and tissue cultures. The dosage of penicillin required for protection, which depends on the host, the route of inoculation, and the strain of virus, varies from a relatively small quantity of 2 to 20 units in the case of infections with murine pneumonitis in the chick embryos (80, 82) to doses of over 2,000 units per day in mice infected intracerebrally with psittacosis and closely related viruses (76, 81). Although the general experience has been that treatment of mice with penicillin merely reduces the infection to a carrier state presumably because of the intracellular situation of the virus, sterilization of infections with the agent of mouse pneumonitis can be brought about with a single dose of 100 units of penicillin (82) in a chick embryo yolk sac. Hamre & Rake (75), working with lymphogranuloma venereum, also observed that following a brief period of growth, penicillin F produced a temporary decrease in the amount of virus in the yolk sac. Penicillins G and K appeared to be merely virustatic; there was no decrease in virus. Penicillin itself is apparently not virucidal *in vitro* except at very high concentrations (75, 82), but certain impurities are. Penicillin probably acts on the virus during multiplication as has been observed with bacteria and may produce analogous alterations in morphology (88).

Penicillin is effective in the treatment of human infections with psittacosis virus (83, 84, 85, 89, 90, 91) but is not useful against clinical lymphogranuloma venereum (86) and is of doubtful value in trachoma and inclusion blennorrhea (87).

Chloramphenicol has shown definite inhibitory activity against several of the Chlamydozoaceae in chick embryos (12, 13, 92, 93, 94), but larger doses were required than for comparable effects against rickettsiae. The results with intracerebral and intranasal infections in mice are not outstanding (73, 92, 93) and appear about equal to those obtained with penicillin.

Aureomycin has been shown to inhibit all of the viruses of the psittacosis-lymphogranuloma group which grow in chick embryos (23, 94) even when 10 million lethal doses of virus per egg were injected. This antibiotic was also effective against psittacosis and lymphogranuloma venereum virus injected intracerebrally into mice, and some activity could be demonstrated when treatment was delayed for as long as 72 hr. This is in contrast to the results of similar experiments with chloramphenicol (92) and penicillin (76, 81) in which partial or doubtful protection was obtained. More recently, quantitative measurements in chick embryos have shown that approximately four times as much aureomycin as chloramphenicol is required to produce the same therapeutic effect (94) against psittacosis, and the indicated ratio for pulmonary infections with other Chlamydozoaceae in mice is probably much larger than this (73). Aureomycin under certain conditions apparently frees the tissues of the mice of the infectious agent (23). Neither aureomycin nor chloramphenicol is significantly virucidal *in vitro*. There is some evidence for the clinical effectiveness of aureomycin against psittacosis (95) and considerable data (96, 97) which indicate a curative effect on acute lymphogranuloma venereum.

Other antibiotics tested against Chlamydozoaceae and found to be inactive include streptomycin as previously mentioned (75, 76) bacitracin (73), and streptothricin (75).

*Sulfonamides.*—Since the discovery of the therapeutic activity of sulfonamides against lymphogranuloma venereum (98), experimental findings have been adequately confirmed by clinical results. Reviews of the earlier literature on this subject with bibliographies will be found elsewhere (75, 99, 110).

Agents of the psittacosis-lymphogranuloma group may be quite sharply divided in two categories, one of definite susceptibility to sulfonamides and one of complete or almost complete resistance as shown in Table I. Strains, such as feline pneumonitis, which are resistant to sulfonamides in one host may be slightly inhibited by large doses in another species (105). In general, sulfonamides have been found to be ineffective against human infections with psittacosis and closely related viruses (106, 107).

TABLE I

SUSCEPTIBILITY OR RESISTANCE OF AGENTS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP TO SULFONAMIDES

| Susceptible | Resistant |
|---|---|
| Lymphogranuloma venereum (75, 80, 81, 99) | Ornothosis (80, 81) |
| Mouse pneumonitis (80, 81, 100, 105) | Meningopneumonitis (80, 89, 100) |
| Psittacosis, 6 BC & Gleason strains (76, 80, 81) | Pneumonitis, Borg and SF strains (80, 81) |
| Trachoma (87, 102, 103) | Psittacosis (other strains) (106 to 109) |
| Inclusion blennorrhea (87, 101) | Feline pneumonitis (104, 105) |

At concentrations of 50 mg. per cent, sulfonamides do not decrease the survival time of psittacosis virus (6 BC) in Maitland tissue cultures under conditions where the virus is not actively growing, although virustatic effects are observed in roller tube tissue cultures where active growth of virus does occur (76). The general experience with agents in this group, with the possible exception of mouse pneumonitis (88), is that sulfonamides are virustatic only (76, 80, 81).

Recent observations have clarified the question of reversal by PAB of the virustatic effect of sulfonamides against Chlamydozoaceae *in vivo* (72, 111, 112). Morgan found that PAB competitively antagonized the chemotherapeutic action of sulfadiazine against the 6 BC strain of psittacosis virus in chick embryos at ratios of 1 part PAB to 500 parts sulfadiazine (111, 112) and also observed reversal of the action of varying doses of sulfadiazine by a fixed dose of pteroylglutamic acid. These observations suggested that sulfonamides inhibited synthesis of pteroylglutamic acid by the virus. Under other experimental conditions where PAB is rapidly excreted, much larger ratios of PAB to sulfadiazine are required. With

the viruses of mouse pneumonitis and lymphogranuloma venereum in experimental pneumonia of mice these ratios were between 800:1 and 200:1, while with these viruses in chick embryos ratios of 4:1 and 1:1 were found (72). Development of over 20-fold resistance to sulfadiazine by the 6 BC strain of psittacosis has been demonstrated (113) by passage in the presence of the drug.

In this section, attention should be called to certain unclassified, virus-like agents which resemble the Chlamydozoaceae in their susceptibility to sulfonamides. These include the agent of heartwater fever of sheep, *Rickettsia ruminantium* (115), which Rake *et al.* (116) believed on morphological evidence to be misclassified although they were unable to show its antigenic relationship to the Chlamydozoaceae. An agent of chick disease producing acute focal lesions in the liver and heart, described by Asplin (114), seems to be a large filtrable virus which is inhibited by sulfonamides with reversal by PAB but apparently not by penicillin.

*Acridines.*—With psittacosis and related viruses in mice and chick embryos, survival rates are increased by treatment with trypaflavine or acriflavine (3,6-diamino-10-methyl-acridinium chloride) (117, 118). Nitroakridin 3582 and other nitroacridines are more active than acriflavine against the agents of psittacosis, feline pneumonitis, lymphogranuloma venereum, and meningopneumonitis (118, 119) in chick embryos and in mice injected by the intranasal or intraperitoneal routes. Atabrine and other chloroacridnes closely related chemically to the above-mentioned nitroacridines except for substitution of a chlorine atom for the nitro group had insignificant inhibitory action (118). These observations indicate that a nitro group at the 3 position or amino groups at the 3 and 6 positions on the acridine ring are essential for this type of antiviral activity.

*Nitrocompounds.*—Nitrobenzene and nitrofuraldehyde derivatives have shown inhibitory activity against the Chlamydozoaceae both in chick embryos and mice (93) which, under certain conditions, as with 5-nitro-2-furaldehyde semicarbazones, equals that of chloramphenicol. The agent of feline pneumonitis, unlike other Chlamydozoaceae, is inhibited by *p*-nitrobenzoic acid or the corresponding acid amide. Nitroaniline derivatives and 2,4-dinitrophenol had no effect (93, 121) on psittacosis and other agents of this group. These observations indicate that several chemically diverse compounds having in common only a nitro group and a ring structure may show significant activity against the Chlamydozoaceae.

*Other substances.*—Moderate inhibitory activity has been observed with *p*-arsenobenzamide in experiments in chick embryos and mice (120). Several enzyme inhibitors have been tested for their effects on psittacosis virus in tissue cultures (121). Growth of this virus was inhibited by *p*-chloromercuribenzoate and iodosobenzoate in concentrations which did not suppress the growth of fibroblasts. These compounds failed to inhibit the growth of virus in embryonated eggs. Enzyme inhibitors found to be inactive at nontoxic concentrations included mono-iodo acetic acid, sodium azide, sodium fluoride, potassium cyanide, and urethane.

### Primary Atypical Pneumonia

Aureomycin exerts a regular and marked beneficial effect on cases of primary atypical pneumonia (122, 123, 124). In a recent controlled study (125), a prompt therapeutic response was observed in 22 patients treated with this antibiotic while in 20 comparable cases treated with penicillin, the duration of the disease was variable and on the average longer. In most of those receiving aureomycin, the symptoms decreased during the first 24 hr. and the fever subsided within 12 to 48 hr.

Aureomycin also shows definite antiviral activity in experimental infections in chick embryos and cotton rats. These studies were done with a virus isolated in 1942 from patients with primary atypical pneumonia (126). This agent should be classed with the larger viruses since ultrafiltration data indicate a particle size over 250 m$\mu$. Chloramphenicol produced only slight inhibitory effects against this virus in cotton rats, but recent unpublished experiments by the author indicate a definite virustatic action in chick embryos. Therapeutic activity of chloramphenicol against human infections is not adequately supported by published data (110).

### Herpes Zoster and Chicken Pox

The agents of herpes zoster and chicken pox both form elementary bodies presumably representing the virus particles, which are large enough to be seen with the ordinary light microscope. Unfortunately, these agents, which may represent a critical intermediate group, cannot be easily studied in the laboratory. Definite evidence of therapeutic effectiveness of aureomycin in human herpes zoster has been presented (127, 128), and there is one report of successful treatment of four cases of this disease with chloramphenicol (129). In cases of chicken pox, topical application of aureomycin ointment to the vesicles caused prompt regression of the lesions while in untreated control areas of the skin of the same patient, the lesions remained or progressed (128). The possibility that these favorable results were due to action of the antibiotic on secondary bacterial infection cannot be excluded.

### Herpes Simplex

Although there have been a few clinical reports of successful treatment with aureomycin of infections believed to be caused by herpes simplex virus (128), experimental evidence in chick embryos and mice contradicts this (23, 130). In infections of the chorioallantoic membrane with this virus, only very slight and irregular effects were observed (130), but the relatively high toxicity of aureomycin for chick embryos prevented the use of doses over 0.5 mg. Thiamine deficiency is reported to prolong slightly the lives of mice infected with herpes simplex virus, but a number of other substances have been tested against this virus with negative results (133).

### The Pox Viruses

All of the compounds mentioned above as being active against rickettsiae or the larger viruses have been tried without success against vaccinia

or other viruses of the pox group (23, 74, 133, 138). Although the pox viruses are only slightly smaller in particle size, they must be quite different biochemically from the Chlamydozoaceae and rickettsiae.

Quinine was reported to decrease the size of fowl pox lesions in chicks (137), but this was not confirmed in the chick embryo (132) or with vaccinia in the rabbit (74). Betaine, choline, and methionine increased the resistance of rabbits to vaccinia (131). It has been pointed out that nonspecific inflammation may, per se, reduce the size of vaccinial lesions in the rabbit skin (74) and various nutritional factors influence susceptibility (197). An impurity contained in commercial penicillin has a marked virucidal action against canary pox, fowl pox, and vaccinia *in vitro* and slightly prolongs the life of chick embryos infected with canary pox (132). Similar effects have been observed with certain substituted allantoins, 1,3-dimethyl uracil and ethyl-n-methyl carbamate (133). In a recent report *p*-aminobenzaldehyde thiosemicarbazone was found to increase the survival time of chick embryos infected by the yolk sac route and of mice infected by the intranasal route with vaccinia virus (140). This compound had no effect on representatives of the rickettsiae, Chlamydozoaceae, and influenza groups.

It should be emphasized that in all the experiments with pox viruses, the effects were slight and not comparable in degree to those discussed in the previous sections. Furthermore, none of the observations have been confirmed independently.

A large variety of substances including certain specific enzyme inhibitors, acridines, quinones, dinitrophenol, benzimidazole, thienyl alanine, substituted uracils, and haloacyl anilides have been reported to inhibit the growth of vaccinia virus in Maitland type tissue cultures (134, 135, 136). The activity of thienyl alanine was reversed when phenylalanine at a concentration 1/20 that of the thienyl alanine was added at the start of the experiment. Similarly, the inhibitory effect of 2,6-diaminopurine is said to be reversible by purines and nucleic acid derivatives (139). Although such experimental procedures may serve a useful purpose, the toxicity of the compounds for the tissue must be considered. The prevention of virus growth may be indirect, nonspecific, or due to death of the tissue. The data on toxicity as presented are too scant to permit a critical evaluation of these experiments. In assessing the significance of such *in vitro* virucidal tests, it should also be remembered that, contrary to expectation, many of the drugs which are inhibitory to the larger viruses or rickettsiae *in vivo* do not show powerful virucidal or rickettsiacidal properties *in vitro*.

## Mumps

Growth of mumps virus and the pneumonia virus of mice (PVM) is inhibited by capsular polysaccharides from Friedlander's bacilli, Types A, B, and C (141, 142, 143). In the experiments with mumps in the allantoic sac of chick embryos, as little as 5 $\mu$g. of polysaccharide was effective, and virustatic effects could be obtained when the treatment was delayed for as long

as four days after infection. Although the polysaccharide prevented absorption of mumps virus by chicken erythrocytes, it had no effect on the absorption of virus by allantoic membrane and no *in vitro* effect upon mumps virus. Evidence was also presented for the presence in certain strains of mumps virus of a variant resistant to inhibition by Friedlander type B polysaccharide (144).

Since these polysaccharides exert no known effect on the growth or metabolism of bacteria, the mechanisms of their action on viruses may well be quite different from those concerned in the bacteriostatic action of chemotherapeutic agents. They are taken up by host cells, fixed in them for long periods (145), and presumably act either on the metabolism of the cells or on the multiplying intracellular virus.

The polysaccharide is unique in being the only agent that is effective against allantoic infections with mumps or influenza when injected by a route different from the virus. Most substances so far tested are active only when put into the allantoic sac, but the polysaccharide is effective by the yolk sac route.

A receptor destroying enzyme from *Vibrio comma* prevents the infection of the allantoic sac with mumps virus (see under INFLUENZA). Aureomycin does not affect the growth of mumps virus in the allantoic sac, but it reduces or completely inhibits the production of hemagglutinin (23) by this virus.

### PNEUMONIA VIRUS OF MICE (PVM)

During the course of observations on the effect of a nonhemolytic *Streptococcus* designated MG on infection with pneumonia virus of mice, Horsfall & McCarty (146) observed that the capsular polysaccharide of this organism had a therapeutic effect. When this substance was given intranasally either before or after infection, the mice survived several fatal doses of the virus, and the growth of the virus was inhibited. Injection of the streptococcus by routes other than intranasal was without effect. Other bacterial polysaccharides including those of the Friedlander bacillus and *Shigella paradysenteriae* as well as the blood group A substance had similar effects. The range of active substances was, therefore, slightly greater than with the mumps virus against which only the Friedlander polysaccharides were active, the inhibitory polysaccharides had no effect on hemagglutination of erythrocytes by PVM and did not reduce the capacity of mouse lung tissue to combine with virus. This evidence and the fact that multiplication of virus could be stopped as late as four days after infection at a stage when many cells were already infected indicated an intracellular site of action rather than an effect on the virus receptors concerned with the initial combination of virus and host cell (145).

The effect of pyridoxine deficiency on susceptibility of mice to PVM has been studied (147). Mice fed a diet deficient in pyridoxine or given desoxypyridoxine during the period after infection were slightly more resistant and

their lung tissues supported less multiplication of virus than was the case with control mice. When pyridoxine deficiency was maintained for eight days or longer before inoculation and continued during the infection, mice became more susceptible to PVM.

### INFLUENZA

*Prophylaxis by blockade of virus host cell combination.*—The concept of cell receptors concerned in the initial stages of infection by influenza virus was introduced by Hirst following his observations on agglutination of erythrocytes by this virus (148, 149). Similarity between reactions of influenza virus and red cells on the one hand and virus and susceptible tissue on the other was emphasized. In each case, the virus was adsorbed and after a short time spontaneously released by a process which is now believed to be an enzymatic destructive action of the virus on the cell receptors. One approach to chemoprophylaxis of influenza is based on attempts to modify the receptors on the host cells or their counterpart on the virus and thus to block the attachment of virus to the cell.

A number of polysaccharides including some of those mentioned in the section on mumps inhibit hemagglutination as do mucoproteins from various sources. One or two of these seem capable of preventing or slowing down virus multiplication in the allantoic sac of chick embryos (150). Noteworthy among these is apple pectin, which, if given in large doses before infection or within 1 hr. afterward, decreases growth of influenza virus as judged by hemagglutination and infectivity titers. Inhibition of the growth of influenza virus by mucoproteins has been observed both in mice and chick embryos (151). Preliminary treatment of the mucoprotein with periodate is said to increase the effect. This is in line with earlier observations that normal ferret sera with high nonspecific inhibitory effect on hemagglutination presumably due to mucoprotein rather than antibody may prevent infection by certain strains (152).

As mentioned above, few of the polysaccharides which inhibit hemagglutination of a virus will inhibit multiplication of the same virus *in vivo*. Certain carbohydrates which are active *in vivo* (146) fail to inhibit hemagglutination or may inhibit hemagglutination but not the adsorption of virus to cells of the chorioallantoic membrane (142). It appears, therefore, that the theory of blockade of host cell receptors by polysaccharide is an oversimplification, that inhibition of hemagglutination is not a reliable model for inhibition of infection, and that in some instances at least polysaccharides may act on intracellular virus rather than preventing attachment of virus to the host cell.

Other experiments involving enzymatic destruction of the receptors suggest the presence of similar chemical groupings on the red cell and the host cell and indicate the role of these receptors in infection (153). An enzyme prepared from *V. comma* destroys the ability of erythrocytes to adsorb influenza virus and thus prevents hemagglutination. Pretreatment with this so-called receptor destroying enzyme also prevents the adsorption of virus to the chorioallantoic membrane of chick embryos and the cells of mouse

lungs. When given intranasally before or soon after the virus, it is effective in preventing or decreasing the severity of infections in mice. It also prevents infection of chick embryos with certain strains of influenza virus, type A, but is less effective against others including the Lee strain of influenza B. When the enzyme is removed from the site of infection in chick embryos or in mice, the tissues again regain their ability to absorb virus and become susceptible to infection. This is interpreted as a regeneration of receptors (153, 154). Metaperiodate, which, at certain concentrations, is said to decrease the elutability of virus from mucoproteins, red cell receptors, and host cells, has an antagonistic action on the protective effect of the cholera enzyme (155), although by itself it does not increase the susceptibility of mice to infection.

*Acridines, dyes, and other substances in the allantoic sac.*—Except for the experiments mentioned above, all attempts to treat or prevent influenza virus infections in mice have been negative (74, 133, 156, 157) or, as in two or three instances to be cited, inconclusive. A number of compounds have been found, however, which undoubtedly exert some effect on the course of infections in the allantoic sac of chick embryos provided they are given by the same route as the virus.

Nitroakridin 3582, which is much more active than other acridines in inhibiting rickettsiae and agents of the psittacosis-lymphogranuloma group, also produces slight and irregular inhibition of the growth of influenza virus, type B (Lee strain) in the allantoic sac of chick embryos (158) when given near the time of infection. Unpublished observations by the writer confirm these results and indicate that a closely related chloroacridine is inactive. Other acridines have been tested for the most part in mice with negative results (74, 133, 157).

Using measurements of the formation of soluble complement-fixing antigen in the chorioallantoic membrane as a criterion of intracellular growth of virus, Hoyle (159) has recently reported that certain triphenylmethane dyes have a retarding effect on the growth of influenza A virus. The virus hemagglutination titer in the treated eggs was also reduced to about half that of the controls. Other dyes produced no effect under the same conditions. In mice Janus green B (160) slightly increased the survival time when given intravenously while others have reported negative results after intraperitoneal injection of this dye (74). Janus green has also been tested in chick embryos with negative results (159).

Hexamidine (4,4-diamidino-diphenoxyhexane), a substance used in the treatment of trypanosomiasis and leishmaniasis, inhibits the growth of influenza A (PR-8 strain) in the allantoic sac of chick embryos (161). The substance was active only when given by the same route as the virus and up to 4 hr. after inoculation. Experiments in mice were negative. The writer has observed similar effects with pentamidine against the Lee strain of influenza B and finds that a more regular inhibition of influenza A and B virus may be obtained in tissue cultures with subtoxic concentrations of pentamidine (162).

Tannic acid and certain extracts of tea also prevent the growth of in-

fluenza virus in the allantoic sac (163, 164). In these experiments, the quantity of tea extract injected into the allantoic fluid apparently produces a concentration of inhibitor *in vivo* equal to or greater than the concentration which is virucidal *in vitro*. Since the prevention of growth occurs only when the substances are injected into the allantoic sac within 2 hr. before or after the virus, a direct virucidal action on the free virus of the inoculum in the allantoic fluid seems quite possible.

With substances such as Nitroakridin 3582 and hexamidine, the low *in vitro* virucidal effects at the concentrations used in the allantoic sac tend to preclude a direct action on free virus in the allantoic fluid. The inhibitory effects may well be due to a local action on the cells which serve as a medium of growth for the influenza virus. That the action is probably not entirely one of simple nonspecific cell toxicity is indicated by the fact that only certain compounds are effective while other closely related drugs of equal toxicity, for example chloroacridines, have no effect. These substances tend to become firmly bound to tissues, and this firmness of combination may be a factor in determining the effect by producing a local virustatic concentration of these drugs that is not attainable by systemic administration as in the treatment of experimental pneumonia in mice. Although these observations may not lead directly to an effective therapy, elucidation of the kinetics of action and metabolic effects of substances such as Nitroakridin 3582 and hexamidine may give valuable clues as to the mechanism of growth of influenza virus.

*Other experiments in mice.*—Unconfirmed reports of slight to moderate activity against influenza infections in mice for quinine (165) and $\gamma$-butyrolactone and related substances (166) will be found in the literature. Negative results with quinine have been reported (74) by other observers. There are no data to indicate whether the observed prolongation of life or diminution in the lung lesions were due to physiological effects of these substances or to inhibition of virus multiplication. In general, slight irregular effects of the kind reported could be expected from a modification of the pathological reaction of the host.

A partial chemoprophylactic effect of atropine given intraperitoneally before intranasal influenza virus infection of mice was attributed to prevention of the accumulation of mucous secretions resulting from ether anesthesia (167). This hypothesis was based on other experimental evidence that preliminary admixture of virus with mucus in the inoculum increased the severity of the infection.

Mercuric chloride and other mercurials exert a reversible inactivating effect on influenza virus *in vitro* (168, 169, 170). Reversal of the inactivation may be produced within certain time limits by sodium thioglycollate or BAL (2,3-dimercaptopropanol) and will also occur in the lungs of mice or in the allantoic sac. Merodiecein (monohydroxy-mercuric-diiodo-resoracein-sulphonphthalein) was stated to have a slight chemoprophylactic effect on influenza infections in mice, but this could not be confirmed by another investigator (171).

Certain dietary deficiencies increase the susceptibility of mice and monkeys to influenza (172 to 175).

### Neurotropic Viruses

Neurotropic viruses of small particle size (10 to 25 mμ.) that have been tested for sensitivity to various chemotherapeutic agents include the encephalitis and equine encephalomyelitis groups, the encephalomyocarditis group, particularly the Columbia MM and SK strains, and the Lansing strain of poliomyelitis. The latter virus is highly neurotropic and will infect regularly only by the intracerebral route. Because of the severity of the intracerebral challenge, a negative chemotherapeutic result does not necessarily exclude activity of a substance against peripheral infection with poliomyelitis or other neurotropic viruses. The Columbia MM and SK strains and certain of the encephalitis viruses readily produce infection by peripheral routes. It is of interest that more suggestive results have been obtained with the latter viruses than with the Lansing strain of poliomyelitis. In most instances, these effects seem to be chemoprophylactic in the nature of a blockade or due to relief of symptoms and pathological reactions.

In earlier work, the chemoprophylaxis of poliomyelitis in the rhesus monkey was accomplished by chemical blockade of the olfactory tissues using alum, zinc sulfate, tannic acid, picric acid, pituitrin S, or other materials (176, 177, 178). These substances produced a coagulation necrosis of the olfactory epithelium followed by sloughing and the formation of a new epithelium which appeared to remain resistant until the time of regeneration of some of the olfactory neurons.

It has not yet been definitely established that intracellular multiplication of the neurotropic viruses can be inhibited by any known substance or procedure. These small viruses may have been reduced by a retrograde evolutionary process to a state of complete metabolic dependence on the host cell. They may be resistant to chemotherapy not so much as a result of their intracellular site of multiplication, but because they lack vulnerable enzyme systems susceptible to direct chemical attack. It should be noted, however, that the presence of complete or partial enzymes has not yet been conclusively demonstrated even in the larger viruses although their presence is inferred from indirect evidence. If this hypothesis is correct, it would be expected that chemoprophylaxis of infections with small viruses would depend largely on changes in the host cells rather than direct action on the virus.

The results of screening experiments with Lansing poliomyelitis in mice have generally been negative (156, 179) although suggestive but unconfirmed results have been reported with pyrimidines (180), certain arsenicals (181), and malononitrile (182). The latter substance was studied because it increased the ribonucleic acid content of nerve cells. When given by the intraperitoneal route before intracerebral infection with small doses of virus, it significantly reduced the mortality. With larger doses of virus or when malononitrile was given after infection, the lives of the treated animals were prolonged, but they died after cessation of treatment. More recently an-

other group of investigators has failed to confirm these results with their Lansing strain and the question of the identity of the virus used in the original studies has been raised (212).

One report indicates a relation of metabolic rate to susceptibility to poliomyelitis. Mice infected with the Lansing strain and receiving large daily doses of thyroid extract or thyroprotein survived several days longer than the controls, while death occurred more rapidly in those receiving thiouracil (183).

Since earlier observations indicated that anesthesia partly counteracted the effects of tetanus and botulinus toxins, studies with ethyl ether and other general anesthetics have been carried out in experimental infection of mice with encephalitis, equine encephalomyelitis, poliomyelitis, or rabies. It was found that several 4-hr. periods of ether anesthesia reduced the mortality rate in mice inoculated with 3 to 6 $LD_{50}$ of St. Louis encephalitis or equine encephalomyelitis viruses, both of which are relatively sensitive to ether *in vitro* (184). No effect was obtained with poliomyelitis or rabies viruses, both of which are resistant to ether. Subsequent attempts to repeat these observations with ether and other general anesthetics against poliomyelitis and equine encephalomyelitis yielded negative results (185).

Material extracted by means of organic solvents from mouse or sheep brain tissue and aged in saline suspension for a period of 4 to 15 weeks significantly lowered the mortality of mice injected intraperitoneally with 1 to 10 $LD_{50}$ of Russian Far East encephalitis virus (186). The extracts were injected intravenously one day before to one day after the virus so that the *in vitro* inactivating effect of these extracts could not have occurred in the peritoneal cavity although the possibliity of virus inactivation in the blood stream should be considered.

Since trypan red and congo red have been observed to partially block the passage of cocaine from the blood into the central nervous system, experiments with these dyes in neurotropic virus infections have been done by several investigators. When the dye was injected intraperitoneally before or together with the MM strain of encephalomyocarditis virus, the mortality of mice infected with 100 $LD_{50}$ was significantly reduced. Repeated intraperitoneal injections of the dye before the virus increased the blockading effect which was said to persist for as long as four weeks (187). No effect was observed after intracerebral injection of virus and intraperitoneal injection of dye, and only very slight or insignificant effects were obtained with virus intraperitoneally and dye orally or subcutaneously (188, 189). The protective effect in the peritoneum has been attributed to a nonspecific local reaction (189). Other dyes did not produce the same effect, and trypan red has no significant *in vitro* virucidal activity at the concentrations used (187).

Darvisul [N-(2-thiazolyl)-phenol sulfonamide] has been reported to have a protective effect in mice against the Columbia SK strain of encephalomyocarditis virus (190). Numerous attempts to repeat these observations have yielded consistently negative results (191 to 194), and no clinical effectiveness against human poliomyelitis could be found (195, 196).

Reviews on the effect of dietary factors on neurotropic virus infections will be found in this volume and elsewhere (197).

## Bacteriophage

The virus-host cell system used in studying bacteriophage seems most closely analogous to the tissue culture or allantoic membrane techniques applied in the study of the animal viruses. In a bacterial culture as in tissue cultures, control of the immediate environment of the host cell is readily accomplished, and the system is uncomplicated by the presence of other factors which, in the intact animal, may indirectly affect the result of experiments in growth inhibition or stimulation. The action on bacteriophage of certain classes of substances which are also active against animal viruses will be reviewed briefly. A more detailed discussion will be found in a recent review by Cohen (198).

Antibiotics such as streptomycin and streptothricin inactivate bacteriophage at concentrations slightly in excess of those that are bactericidal. Bacteriostatic or bactericidal concentrations inhibit both host cell and virus (199). Penicillin is of interest in that it does not inactivate or prevent growth of bacteriophage although used in concentrations which are bacteriostatic to the host cell (198 to 201). With staphylococci, the speed of lysis in the presence of both bacteriophage and penicillin may be increased although the growth of phage does not proceed at the normal rate. Other observers have reported increased growth of bacteriophage under certain conditions (201, 202) in the presence of penicillin.

Bacteriophage has been used in experiments to find antibiotics which might be active against other viruses. A useful paper disc assay method which readily distinguishes between activity against bacteriophage alone, combined effect against bacteriophage and bacteria, or antibacterial action only (203, 204), has led to the isolation of two antiphage antibiotics from an *Aspergillus*. Neither of these antibiotics shows significant bacteriostatic or bactericidal activity, and in one report (203), it is demonstrated that the agent has no *in vitro* activity against bacteriophage, does not affect bacterial cells so as to render them permanently unsuitable for growth of bacteriophage, but apparently inhibits intracellular multiplication of the virus.

Earlier work on inhibition of bacteriophage by bacterial extracts and polysaccharides from phage-susceptible species of organisms (205, 206) is quite analogous to recent studies on receptor blockade of influenza and mumps viruses with carbohydrate complexes (142, 150, 151). Inactivation of bacteriophage by polysaccharides of nonbacterial origin has also been reported (207).

Antimetabolites and specific enzyme inhibitors such as 5-methyl tryptophane, methionine sulfoxide, cyanide, iodoacetate, and sulfathiazole when used at concentrations which inhibit growth of phage on infected cells also inhibit bacterial multiplication or are bactericidal. A variety of modes of action have been postulated, and these include reversible or irreversible action on the bacteriophage itself, prevention of adsorption or penetration of

phage on the bacterial cell, metabolic effects on synthesis of coenzymes, proteins or nucleic acids, and prevention of lysis (198). When growth or metabolism of the host cell is inhibited, indirect effects are possible, and these seem to complicate the interpretation of the results as much as they do in tissue culture experiments.

The action of acridines on bacteriophage is of special interest because of the apparent inhibitory effects of substances of this class on other viruses and also because these compounds have served as a tool to gain additional knowledge about growth of bacteriophage. Acriflavine, proflavine, and phosphine GRN, when used in nonbacteriostatic concentrations, prevent the lysis of phage infected suspensions of *Escherichia coli* (208, 209, 210). Sensitivity to acridines varies with the phage type and can be altered by mutation (211). Certain bacteriophages for *Micrococcus pyogenes* var. *aureus* are not inhibited by any concentration of phosphine GRN below the bacteriostatic level (209). Yeast nucleic acid neturalizes the antiphage activity of acridines (208, 209), and similar effects have been described for riboflavin, thiamin, nicotinamide, and sodium lauryl sulfate. From the experiental data now available, it is not clear as to which of these reversal effects are due to simple *in vitro* combination with the acridine and which of them may be attributable to metabolic antagonism.

Proflavine inhibits the intracellular growth of bacteriophage and appears to block a late reaction necessary for the production of active phage. Complete inhibition of growth was obtained when drug was added up to 15 min. after infection. Removal of proflavine from the phage-bacterium system early in the course of the virus growth permits phage liberation without delay, and the amount liberated decreases progressively with lengthening of the interval between infection and removal of proflavine. Proflavine had no appreciable effect on infectivity when mixed with bacteriophage at the concentrations used in the experiments. It is concluded that some of the earlier phases of phage synthesis are completed in the presence of the drug and that the phase of formation of infectious particles in the last phase may be the step which is blocked (211). In these experiments, the proflavine failed to inhibit lysis of the bacteria even when no active phage particles were formed. This seems inconsistent with the results reported above for acriflavine and phosphine GRN (208, 210) but may be due to differences in the concentrations of drug used by the various investigators. Presumably noninfective virus particles appear in the medium as a result of this lysis in the presence of proflavine, but their actual presence was not demonstrated.

It is apparent that considerable fundamental knowledge of the mode of action of chemotherapeutic agents is coming from the studies with bacteriophage and this may point the way to similar methods of approach with the animal viruses. Although a number of substances influence the growth of rickettsiae and the animal viruses under experimental conditions, very little is known at present about their mode of action. Some advances have been made with the rickettsiae and with the mumps and influenza group. The present approach to chemotherapy of virus dieases is predominantly empirical but further elucidation of the nature of virus growth and metabolism should

parallel this intensive search for active substances. In fact, it may be necessary to know much more about the way in which viruses grow and to solve their relation to the fundamental life processes of cells before a successful solution of the problem of virus chemotherapy can be devised.

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# ANTIBIOSIS IN RELATION TO PLANT DISEASES

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Pioneers of microbiology and plant pathology (1, 2) were well aware of antagonistic phenomena among microorganisms and their relation to plant disease. But as Fawcett (3) pointed out, effects of known mixtures of cultures were rarely studied in detail, because pathologists were too deeply impressed by the need of pure culture work which is basic for exploring host-pathogen relationships. Recently, however, studies of antibiosis in relation to plant pathology have received a powerful stimulus; antibiotic substances have become a major factor in the treatment of many human diseases. A vast fermentation industry has sprung up to manufacture the "wonder drugs" of lowly microbial origin. For the searcher after remedies of diseases of humans, animals, or plants, the metabolic products of thousands of fungi, actinomycetes, and bacterial species are providing a new field for exploration.

Waksman (4) defined antibiotics as substances produced by microorganisms and capable of inhibiting or destroying other microorganisms; but related substances of higher-plant origin will be mentioned here also. The terms "antibiosis" and "antagonism" have been employed interchangeably in plant pathological literature, and they will be used so here. Antibiosis, then, will refer to all inhibitory or destructive effects of microorganisms on one another, whether defined antibiotic substances are involved or not; it is a collective term that covers widely differing kinds of relations.

This review will discuss antibiosis and antibiotics in relation to plant pathogens and disease control. Various aspects of this field have been treated by other reviews (4 to 8). Whatever the true nature of viruses, their behavior as etiological agents of plant diseases is so similar to that of known microorganisms that they may properly be considered in a section of this article.

## Pathogens Affected by Antibiotic Microorganisms

*Fungi: obligate parasites.*—Powdery mildew and rust fungi are frequently parasitized by other fungi. *Cicinnobolus cesatii* is a common parasite on many mildew species. Emmons (9) made a study of its parasitism on *Erysiphe cichoracearum*. The fungus penetrates the mycelia of its host, and forms pycnidial spores within the empty fruiting bodies of the mildew. Yarwood (10) determined the moisture relations of *C. cesatii* and *Erysiphe polygoni*. The spores of the parasite required free water for dissemination and germination, the spores of the mildew did not.

McAlpine (11) found the universally-occurring hyperparasite, *Darluca filum*, on 24 per cent of the known rust species of Australia. Keener (12) came to the conclusion that biological races of the parasite attack certain rust species but not others. *Darluca* parasitized uredospores more frequently than aecia and teleutospores. Adams (13) discovered it on aecia of the hemlock rust. The *Darluca* disorganizes the mycelium of the rust and suppresses spore formation. Another rust parasite, *Tuberculina maxima*, the "purple mold," attacks the destructive white pine blister rust. Huber (14) observed that *Tuberculina* destroyed aecia and also the bark beneath aecial and pycnial structures, and thus prevented aecia formation the following year. Various fungi and bacteria affect cereal rusts (15, 16). The fungi belong to the imperfecti, *Fusarium*, *Verticillium*, *Cephalosporium*, and *Trichoderma*. Smith (17) discovered a *Cladosporium* sp. parasitic on asparagus rust in California. This parasite uses wind-borne rust spores as vehicles for distribution. Frequently it develops so abundantly that it suppresses the rust disease in the field.

*Soil-borne fungi.*—Certain strongly antibiotic organisms have developed peculiar parasitic habits toward some soil-borne pathogens. Greaney & Machacek (18) studied the antagonistic action of *Cephalothecium roseum* against *Helminthosporium sativum* in culture and in soil, while Koch (19) described its parasitic behavior on stromata of the plum pathogen, *Dibotryon morbosum*. Other examples are *Trichoderma* spp. (20), *Gliocladium* sp. (21), and *Papulospora* sp. (22), which inhibit a wide variety of pathogenic fungi and actinomycetes, and which frequently twist their hyphae around those of *Rhizoctonia solani* in some form of parasitic action.

In nature, however, these and other antagonists occur and function usually within the complex dynamic mixture of soil fungi, bacteria, and actinomycetes. Concepts and principles of such antibiotic effects were developed by Sanford (23), Henry (24), and Garrett (25), mainly through their work on cereal root rot diseases. Their conclusions will be summarized in the following three paragraphs, as a basis for the discussion of other contributions.

(*a*) Plant pathogenic fungi differ in their susceptibility to the antibiotic action of the soil microflora. *Ophiobolus graminis*, for example, is more sensitive than *H. sativum* and *Fusarium culmorum* (23). Susceptible pathogens have been said to "escape" the competition of other soil organisms (25). Increasing sensitivity to microbial antagonism has thus been linked with evolution from saprophytic ("soil-inhabitant") to predominantly parasitic habit ("soil invader").

(*b*) The resting phase and the saprophytic phase of "soil-invading" pathogens are the most vulnerable phases of their life cycle. In many cases antibiotic action is simple destruction by rotting. Antibiotic activities of the soil microflora are dependent, therefore, on readily available energy for decomposition processes (25).

(*c*) Antibiotic action depends upon the "right" environmental conditions; e.g., Henry (24) discovered that at high temperatures seedling infec-

tion by *O. graminis* was suppressed in natural soil (when compared with sterilized soil); at low temperatures infection was about equal in nonsterilized and sterilized soils.

With regard to the effects of environmental conditions there is a parallel, but only in part, between the antibiotic action of complex soil microfloras and that of individual soil organisms. Sanford & Broadfoot (26) and Slagg & Fellows (27) have studied *O. graminis* intensively, in association with many fungi, bacteria, and actinomycetes isolated from soil. They concluded that an individual organism may inhibit the pathogen on one culture medium, stimulate its growth on another, and may or may not have antibiotic effects in soil; the same holds for culture filtrates. Anwar (28) has reported an example of correlation between antibiotic action of the soil microflora and that of its constituent members. In natural soil, *H. sativum* diminished rapidly, while during the same period *Fusarium lini* did not; its population followed the seasonal variation of the soil microflora. In culture, the number of microbial isolates (from this soil) that inhibited *H. sativum* was larger than the number that affected *F. lini*. Similar results were obtained by culture filtrates of the isolates.

Another aspect of antibiotic phenomena has recently been stressed by Simmonds (29) and Ledingham *et al.* (30). Working with *H. sativum* they found that wheat seed, culms, and stubble have a characteristic surface flora (capable of antibiotic action) that differs from the soil microflora. This surface flora is considered to be very important when evaluating seed treatments, or when testing seedlings for varietal resistance to the disease. Along a similar line, Eaton & Rigler (31) discovered that the assumed "immunity" of corn seedlings to *Phymatotrichum omnivorum* was due to the protective action of antibiotic microorganisms associated with the roots in soil; in sterilized sand, the pathogen attacked the roots and killed the seedlings. The same authors were able to increase resistance of cotton roots to *P. omnivorum* by treatments that raised the carbohydrate content of the root bark. This was connected with notable increases in the number of blue-green fluorescent bacteria, and it was suggested that they were responsible for the protective effect against the disease.

Some seed-borne pathogens are susceptible to antibiotic action of the soil microflora, others seem to be little affected. Most likely, internally infected seed is beyond protection, and pathogens lodging beneath the seed coat will not be easily reached by the effects of other organisms. Christensen (32) showed that barley seed, naturally infested by *H. sativum*, was not protected against disease by those organisms which gave partial protection to artificially inoculated seed. Henry & Campbell (33) found this to be true also for cereal smuts, but they noted that seedling diseases of flax were suppressed in nonsterilized as compared with sterilized soil.

Phycomycetous pathogens are difficult to separate from "contaminating" soil bacteria. Drechsler (34) holds that, in soil, putrefactive bacteria restrict *Pythium* and *Phytophthora* spp. by debilitating their mycelia; that parasitic chytrids prevent oospore formation; and that amoeba, rhizopods, and para-

sitic hyphomycetes help in soil sanitation. He isolated and described two of the fungi parasitic on *Pythium*. One, a *Dactylella*, is related to nematode-capturing forms that have been described in a monograph by Drechsler (35).

*Bacterial plant pathogens.*—Many students of bacterial pathogens have recorded incidental observations on antibiotic phenomena in culture or soil. Loede (36), for example, found in 1902 that certain micrococci were strongly antagonistic against *Agrobacterium tumefaciens*. Ark & Hunt (37) reported the isolation from soil of *B. vulgatus* and of another bacillus which were highly antagonistic when tested in culture against 16 representative species of plant pathogenic bacteria. Landerkin & Lochhead (38) made a systematic survey of 50 actinomycetes isolated from soil. In general, the strongest antagonists were the most versatile i.e., they affected the largest number of bacterial pathogens.

Ecological relations of pathogenic bacteria to the general soil microflora may be similar, in some ways, to those of pathogenic fungi discussed above, but there is little more than circumstantial evidence to prove this point. Diachun & Valleau (39) determined by means of an ingenuous leaf inoculation test that *Bacterium tabacum* and *B. angulatum* overwinter and persist readily in natural soils, apparently on the roots of various plants on which these bacteria do not cause disease. Many plant pathogenic bacteria do not persist or overwinter readily in soil, though they are known to remain viable for long periods in sterilized soil (40, 41). Apparently they are susceptible to the inimical effects of the soil microflora, while other bacterial pathogens, such as *A. tumefaciens*, survive in soil because they are resistant.

The frequently reported association of bacteriophages with the etiological agents in diseased plants suggests a relationship which may be beneficial for the plant. Thomas (42) for example, isolated phage for *Bacterium stewartii* from tissues of corn plants killed by the disease and from those of plants which later recovered. Phage was also isolated from the grain of a badly infected crop which, when planted, developed only 5 per cent infected plants.

## Control of Plant Diseases by Exploiting Antibiosis

The preceding sections have illustrated the diversity of antibiotic manifestations against plant pathogens. Exploitation of antibiosis for disease control must be based therefore on investigation of the individual diseases, correlating etiological and ecological relations of the pathogen.

*Diseases caused by obligate parasites.*—Artificial biological control may be visualized as proceeding in two ways from the understanding of natural biological control: (*a*) by increasing the antibiotic microbial population so that it acts as a protective barrier around suceptible organs of the host plant, and (*b*) by reducing the inoculum potential of the pathogen, inducing as it were an epidemic in the population of the pathogen. The latter is the classical approach in biological control of insects, by predators or parasites. This method has been tried on parasites of rusts and mildews. Tubeuf (43) reported success in controlling blister rust of pines in Germany. He placed spores of the parasite *Tuberculina maxima* on aecial fruiting structures of

the rust fungus, at strategic spots of infested woods. Tubeuf claimed that the disease was greatly reduced by preventing spore formation and spread of the pathogen, the hyperparasite being disseminated by wind to other infected trees. Hubert (14) attempted to distribute the *Tuberculina* in the Pacific Northwest, but only a few of his inoculations on *Cronartium ribicola* were successful. He attributed the failure to weather conditions unfavorable for the spread of the hyperparasite.

*Soil-borne diseases: soil or seed inoculation.*—Soil inoculation with specific organisms presupposes that the inoculant will successfully compete with the existing microflora, and that it will be able to exert its antagonistic action against the pathogen in the soil as it did *in vitro* (44, 45, 46). Lochhead & Landerkin (47) have recently shown that of 11 soil actinomycetes inhibiting *Streptomyces scabies*, eight were capable of inhibiting each other, a situation which may be magnified enormously in soil. Most workers in the field of antibiosis have demonstrated antagonism with pure cultures in agar media or in sterilized soil. When disease control by specific antagonists was attempted in nonsterile soil, results were variable or even contradictory. For instance, *Trichoderma* spp. were reported to give partial control of damping-off of vegetable seedlings (48), variable control of *Rhizoctonia* damping-off of citrus seedlings (49), and no control of potato scab (50). Apparently, specific antagonists inhibit certain pathogens under some conditions but not under others, and in some soils but not in others.

Seed treatment with specific organisms is another direct approach to biological control of plant pathogens. Jensen (51) has reviewed critically the extensive publications of Russian investigators who are enthusiastic over "seed bacterization," that is, application of bacterial preparations to nonleguminous seeds. He came to the conclusion that "the effects of bacterization (on crop yields) may on the whole be fictitious." However, he considers it to hold out promise for preventing infection by plant pathogens, as a result of temporary changes in the microfloral surroundings of the seedlings. Recently Ledingham *et al.* (30) have reported from Canada success in experimental bacterization of wheat seed against *H. sativum* infection.

*Soil-borne diseases: soil modification.*—Essentially this approach involves treating soil—by incorporation of organic matter or changing its reaction—to encourage quantitative and qualitative changes in the saprophytic flora which will adversely affect the soil-borne plant pathogen. Cereal root rot diseases, especially the take-all disease caused by *Ophiobolus graminis*, are kept in check by practices such as fallow, crop rotation, and applying organic manures. Garrett (52, 53), Fellows (54), Sanford (55), and Clark (56) suggested that success of these measures is due, at least in part, to their beneficial effect on antibiotic organisms which suppress the saprophytic phase of the pathogens. The cotton root-rot disease seems to yield to practices that involve biological control. In Arizona, King (57) had spectacular success by repeated manuring of experimental cotton plots. In Texas, Lyle *et al.* (58) found that sweet clover rotations offered the best practical control yet devised for holding the cotton root-rot disease in check. They suggested

antibiotic effects as a partial reason. Mitchell *et al.* (59) reduced cotton root rot in field experiments by terminating the parasitic phase of the pathogen through early fall plowing. Simultaneous addition of cow manure aided in microbial destruction of the saprophytic growth of *P. omnivorum* and of its sclerotical resting bodies.

Mitchell *et al.* (59) proved that the way toward better practical control measures may be paved by an understanding of the mechanism of biological control. By means of the Cholodny slide technique, they obtained direct microscopic evidence that mycelia and sclerotia of *P. omnivorum* were decomposed in manured soil by successive groups of soil microorganisms. No evidence was presented that antibiotic substances were active. The actual role of antibiotics in complex mass action is not clear. They may be active in micro-environments; but the idea of mass production, or of accumulation of antibiotics in the soil, has met with considerable skepticism (4). Another mechanism of biological control was suggested by Garrett (60), namely, that adding organic matter depressed the parasitic action of *O. graminis* because carbon dioxide was increased in the micro-atmosphere around the roots.

The microflora of the "rhizosphere" may also play a significant role in biological control of soil-borne diseases. This microflora, in the immediate vicinity of roots of growing plants, differs quantitatively and qualitatively from the indigenous flora of the surrounding soil. Moreover, the balance of its constituent groups can be altered by treatment. Hildebrand & West (61) found that root rot of strawberry could be controlled by incorporation of soybeans and glucose but not of red clover. In the rhizosphere of healthy plants, non-pathogenic fungi and bacteria replaced potentially pathogenic forms such as *Fusarium, Rhizoctonia,* and *Cylindrocarpon,* which occurred on diseased roots. Essentially similar results were obtained by Katznelson & Richardson (62) with both acetic acid and dried blood. Most recently, Rouatt & Atkinson (63) demonstrated that soybeans incorporated into potato-scab soil exerted a controlling influence. Previously, Lochhead & Landerkin (47) reported that the rhizospheres of potatoes in "soybean soil" contained significantly larger numbers of actinomycetes antagonistic to *Streptomyces scabies* than the rhizospheres of tubers in the untreated soil. It would appear, therefore, that the establishment by appropriate soil amendments of a rhizosphere microflora "inhospitable" to a particular pathogen is a very important approach to biological control and one which should be given much more extensive trial.

*Bacterial diseases.*—Phages for many phytopathogenic bacteria have been isolated, but there is too little experimental evidence to warrant generalization on phage therapy in plants. The possibility of reducing bacterial infection and resulting losses by this means cannot be dismissed. Katznelson (64) has pointed out that various factors will have to be considered where phage therapy or prophylaxis is contemplated: development of phage-resistant organisms (which may be very rapid with some species such as *Pseudomonas phaseolicola*), absorption and inactivation of phage by plant

tissues, modification of environmental conditions and soil, and application of methods such as seed treatment or spraying.

Thomas (42) reduced infection of corn seedlings by *Bacterium stewartii* from 18 to 0.4 per cent, by treating inoculated seed with phage. Israilsky (65, 66) treated the roots, stems, and seeds of beets with phage before inoculating with *Phytomonas tumefaciens* and found the percentage infection much reduced. The phage of *Bacterium mori* had a prophylactic effect against infection of mulberry leaves (67). Novikova (68, 69) observed that during the waning of an epiphytotic of wild fire there was an increased incidence of phage in the leaves of infected plants. Sprinkling test plots with this phage resulted in a decrease of wild fire infection by 50 per cent.

## Antibiotic Substances in Relation to Plant Diseases

Since the advent of penicillin and the isolation of many antibiotics, investigators can deal with pure (or nearly pure) chemicals of constant composition, instead of culture filtrates of unknown, variable composition. This improvement in methodology has resulted in three important discoveries: (*a*) that the same antibiotic may be produced by several unrelated microorganisms; (*b*) that several antibiotics may be excreted by a single organism; and (*c*) that many antagonistic organisms produce growth-inhibiting and growth-promoting materials.

*Effect of antibiotics on plant pathogens in culture.*—Most of the known antibiotics have now been tested on phytopathogenic bacteria and fungi. The results are often difficult to compare, because they vary considerably depending upon method of testing, test organism, antibiotic, and its stability. Waksman *et al.* (70) tested six substances against nine plant pathogenic bacteria, employing agar cup diffusion and streak dilution methods. The antibiotics exhibited marked specificity. The activity of streptothricin ranged from 5,000 dilution units for *Bacterium solanacearum* to 150,000 for *Agrobacterium tumefaciens*. Fumigacin and chaetomin inhibited some of the pathogens but not others. Gilliver (71) tested 13 antibiotic substances in broth culture against 13 bacterial and 19 fungal species, mostly plant pathogens. Contrasting gram positive to gram negative bacteria, the former were generally the more sensitive to proactinomycin, mycophenolic acid, penicillin, tyrothricin, and gliotoxin; about equally sensitive to aspergillic acid and clavacin; and less sensitive to penicillic acid. Katznelson & Sutton (72) compared the effect of 10 antibiotics on strains of *Pseudomonas phaseolicola* and *Xanthomonas phaseoli*. Polymyxin and aureomycin were the most potent, penicillin the least potent. Most of the other studies were restricted to single antibiotics in relation to plant pathogenic bacteria, such as Ark's (73) report on the spectrum of streptomycin. Striking variations in strain responses were observed by De Ropp (74) who exposed six strains of *A. tumefaciens* to seven antibiotics. The order of activity was about the same for all strains; the most active antibiotics were aureomycin, chloromycetin, and streptomycin.

Few specific antifungal substances have been isolated. Apparently the

search for them has not been as intensive as for antibacterial substances of potential medicinal value. Some antibiotics are active against certain plant pathogenic fungi and bacteria, e.g., gliotoxin, aspergillic acid, mycophenolic acid, and tyrothricine [Gilliver (71)]. Brian and his associates (75 to 78) isolated four antibiotics that show activity against plant pathogenic fungi, but little or no activity against bacteria. In spore germination tests the three most sensitive organisms (*Botrytis allii*, *Fusarium coeruleum*, and *Penicillium digitatum*) were inhibited by gliotoxin at 1 to 30 $\mu$g. per cc. (79); by viridin at 0.003 to 0.2 $\mu$g. per cc. (76); by gladiolic acid at 4 to 16 $\mu$g. per cc. (75); and by glutinosin at 0.8 to 25 $\mu$g. per cc. (77). Two new antifungal substances have been extensively tested. Whiffen (80) obtained inhibition by actidione (from *S. griseus*) of 33 species and strains of fungi, at 0.125–100 $\mu$g. per cc. Leben & Keitt (81) tested antimycin in partially purified form on many species of plant pathogenic fungi. Inhibition ranged from 0.4 $\mu$g. per cc. (*Venturia inequalis*) to 250 $\mu$g. per cc. (for *Pythium* sp.). Variations in strain response were noted for actidione and antimycin.

Extensive surveys of higher plants have been made in search for antibacterial and antifungal substances. Hayes (82), for instance, observed that inhibitory activities varied greatly with factors affecting the plants, with conditions of extraction, and with bacterial species. Substances from *Allium* spp. were most effective against *A. tumefaciens*, those from *Convolvulus arvensis* against *Erwinia carotovora*.

*Antibiotics and plant disease control.*—For practical disease control, antibiotics have to compete with other pesticides, with regard to effectiveness, nontoxicity to plants, and cost. Few extensive field trials have been attempted so far. However, interesting possibilities have emerged from experimental work. Goodman & Henry (83) reduced infection of barley by *Xanthomonas translucens* by means of subtilin. Seedling emergence and protection were much better when subtilin was applied with the bacterial inoculum rather than some days later. Ark (73) reported control of seed-borne bacterial pathogens of cucumber and tomato, by soaking seed in streptomycin solutions. Control of bacterial ring rot of potato was obtained in experimental treatments of seed pieces with streptomycin, but not with penicillin (84).

Leben & Keitt (85) concluded that antimycin has promise as a protectant fungicide. They tested it on apple scab and tomato early blight in careful laboratory and greenhouse studies. Crude preparations of the antibiotic were nontoxic when sprayed onto leaves, and they were compatible when mixed with commonly used insecticides. Actidione has been hailed as having potential value as a fungicide, but recent reports on its plant toxicity have made its usefulness questionable (86).

*Antibiotics and soil-borne plant diseases.*—Most antibiotics have been isolated from soil organisms. This raises the question: What role do these substances play in natural soils, in relation to plant pathogens and plant diseases? Brian (87) suggests that antibiotics have considerable ecological significance in soil processes. Probably many antagonistic organisms find

natural conditions favorable for the production of antibiotics in minute quantities, and these are likely to be effective in close contact with plant pathogens. There is no evidence, however, that microbial antagonism in soil can be attributed primarily to antibiotic substances or that they accumulate in soil, except under special conditions. Waksman & Woodruff (88) extracted actinomycin-like materials from poor, but not from good soils. Growth failure of conifers in poor heath soil was attributed by Brian *et al.* (89) to toxic excretions of the dominant microflora (*Penicillium* spp.) which inhibited mycorrhiza formation.

The production and persistence of individual antibiotics in soil depends on numerous factors, especially on their stability, on adsorption, and on their resistance to processes of decomposition activated by the soil microflora. Actinomycin was reported to disappear more rapidly from nonsterilized than from sterilized soil (88). Grossbard (90) was able to extract measurable amounts of clavacin from sterilized soil inoculated with *Penicillium patulum*, if glucose or wheat straw were added to the soil. The clavacin was still extractable after one week, but not after six weeks, from soil which was not protected from recolonization with microorganisms.

Gottlieb & Siminoff (91) incorporated clavacin and streptomycin into sterilized soil, which was then inoculated with *B. subtilis*. Small amounts of clavacin suppressed *B. subtilis;* but streptomycin, though effective *in vitro* at high dilutions, did not inhibit the test organism in soil, even when applied in large amounts. Further experiments showed that the streptomycin was readily adsorbed on clay, while clavacin was not adsorbed. Moreover, a mutant of *Streptomyces griseus* incapable of producing streptomycin, was as effective as the original *S. griseus* in suppressing *B. subtilis* in sterilized soil. Thus, the effects of antagonistic soil organisms are not necessarily connected with their ability to produce antibiotics. When one considers that Gottlieb & Siminoff (91) obtained their data under controlled conditions by the use of sterilized soil, it becomes obvious that challenging and complex problems await investigators probing into the role of antibiotics in relation to plant pathogens in natural soil.

## Antibiosis in Relation to Virus Diseases

*Action of microorganisms on viruses.*—As early as 1886, Mayer (92) working with tobacco mosaic disease, suspected the soil of harboring virus. Mulvania (93, 94), Johnson & Ogden (95), and Hoggan & Johnson (96) found that aerobic conditions accelerated biologic activity and hastened the removal of virus from the soil. Laboratory studies were soon designed to determine the direct effect of pure cultures of various microorganisms on sterile virus extract or infected tissue.

Mulvania tested 11 bacterial species and obtained a marked reduction in virus infectivity with cultures of *Proteus vulgaris* and *Aerobacter aerogenes*. Johnson & Hoggan (97) surveyed a field of some 90 species of bacteria and fungi and found many of them capable of decreasing virus infectivity, suggesting that "the microorganisms most likely utilized the virus constituents

in their metabolism." The notion persisted rather generally that the antagonistic microorganism must be brought into close contact with the virus in order to bring about its destruction. However, these authors included a few tests in which fungi were grown on liquid media containing tobacco mosaic virus extract. Certain fungi which proliferated only on the surface of the liquid, rendered the virus throughout the liquid noninfective, thus foreshadowing the concept of an inhibitory substance being excreted by the fungus into the surrounding medium. Goldin (98) reported that the yeast, *Torula kefiri*, inactivated a sterile filtrate of tobacco mosaic virus almost completely in two days under aerobic conditions, while some putrefactive bacteria proved incapable of inactivating or multiplying on purified crystalline virus.

Johnson (99) studied the properties of inhibitors from cultures of *A. aerogenes* and *Aspergillus niger*. He noted that the inactivation was too rapid to be due to the slower process of decomposition. Johnson (100) then devised a culture technique whereby most of the inactivator could be removed from the inoculum, and obtained a reactivation of tobacco mosaic virus which led to the postulation that "a relatively loose specific molecular union" had taken place. When growth products of *A. aerogenes* were applied to a local lesion host before inoculation with tobacco mosaic virus, the plant was protected from infection for as long as a week. Culture filtrates of this organism also inhibited four other viruses. Fulton (101) investigated the nature of various inhibitors, among them the growth products of *A. niger*, in both systemic and local lesion hosts, and obtained inactivation of tobacco mosaic, and other viruses. Ramon, Manil & Remy (102) reported a differential effect of the culture filtrates of *B. subtilis* on the viruses of tobacco mosaic and tobacco necrosis. The former was unaffected but the latter was inhibited. Price *et al.* (103) tested the filtrates of 40 species of fungi for their ability to inhibit the viruses of southern bean mosaic, tobacco mosaic, and tobacco ringspot. Three of the fungi, *Trichothecium roseum*, *Neurospora sitophial*, and *N. crassea* inhibited all three viruses immediately but the reaction was reversible upon dilution. The inhibiting filtrate was effective when rubbed on the plant before or up to 30 min. after inoculation with virus. Utech & Johnson (104) investigated 94 species of bacteria and fungi in their search for virus inhibitors. Tobacco mosaic virus was generally employed, but a few tests on other viruses were made with considerable success. There was widespread occurrence of inhibition among all of the classes of fungi. Price & Gupta (105) studied the effect of growth products of 49 species of fungi and found that 10 of them reduced the infectivity of southern bean mosaic, tobacco mosaic, and tobacco necrosis viruses, the degree of reduction varying with the host.

*Action of antibiotics on viruses.*—Manil (106, 107) reports failure in the control of purified tobacco mosaic virus with penicillin, streptomycin, tyrothricin, actinomycin, and actinomycetin. Mixing the antibiotics with virus *in vitro*, or pretreating the leaves of a plant with them, or allowing the plant to absorb them, did not protect from infection with virus. Beale

& Jones (108) introduced large amounts of various purified antibiotics into the vascular system of *Nicotiana tobacum* and *N. rustica*, systemic hosts of tobacco mosaic and potato yellow dwarf viruses, respectively. Penicillin, streptomycin, aureomycin, chloromycetin, and terramycin were tested in systemic hosts, and also mixtures of tobacco mosaic virus and the antibiotics in local lesion hosts. No inhibition of the virus was noted.

No antibiotic has yet been found capable of controlling a plant virus disease. Since the plant viruses are intracellular and multiply in a manner similar to obligate parasites, a logical approach to an effective method of control would seem to be some form of internal therapy. It is hoped that as the search for new antibiotics continues unabated some will prove to be valuable in the chemotherapeutic control of plant viruses.

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# IMMUNOLOGICAL REACTIONS IN VIRAL DISEASES

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If biologically active material is introduced either naturally or artificially into a host, the latter will react with certain changes which, in many instances, are recognizable as characteristic responses to a given organism and thus may be used for the identification of the causative agent. Viruses, as biologically active materials, fit well into this category. The aim of this article is to describe one aspect of host reaction to viral agents, the so-called immunological response, which may be manifested by the appearance of immune bodies detectable by means of several tests currently employed. However, at least four symposia, published in the recent past (1 to 4), cover the same field quite extensively. Furthermore, because of limitations of space, the scope of this review will be restricted to selected publications of the last three years which have not been discussed in the above studies, and which at the same time appear to contribute materially to the subject under survey.

## The Neutralization Test

*Concept.*—The neutralization test is based upon the reaction of a susceptible host to inoculation of a mixture of serum and living virus.

*Applicability.*—(*a*) For diagnostic purposes: (i) detection of specific antibodies in the sera of persons or animals, obtained during the acute or convalescent phase of a suspected viral disease; (ii) for identification of the causative viral agent. (*b*) For the study of antigenic relationships between different viruses. (*c*) For the determination of potency of antiviral sera or vaccines.

*Technique.*—Because of the nature of the test, it is apparent that there can be no standard procedure applicable to all viruses. Basically there are two procedures; in one serial dilutions of virus are mixed with undiluted serum, and in the other serial dilutions of serum are mixed with one, usually predetermined, dilution of virus. These mixtures, before inoculation into susceptible animals, are incubated, the temperature and period of incubation being dependent on the test material.

*Viral encephalitides.*—The neutralization test has been most widely used and has probably found its greatest application in this type of infection. The evaluation of the results has also been critically scrutinized [Hammon (2)], and an attempt at standardization has been made by the Commission on Neurotropic Virus Diseases of the U. S. Army (3).

Techniques of the tests have been described in detail by Hammon (2) and by Olitsky & Casals (5). Certain factors which must be taken into con-

sideration have been analyzed in detail in various publications. That the conditions of storage of test sera seem to play an important role in the results obtained was indicated by Morgan (6) and Whitman (7) who found that the neutralizing ability of western equine encephalomyelitis antiserum remains unaltered if sera are kept frozen at −70°C., but falls off if sera are held for prolonged periods at 4°C., or are inactivated at 56°C. The neutralizing capacity of such sera can be restored by addition of complement. Dozois *et al.* (8) observed that the C′2, C′3 and C′4 components are of greater importance for enhancing the neutralizing action of heated immune serum than the C′1 component.

The time of contact and temperature of incubation of serum-virus mixtures prior to inoculation into the susceptible host apparently also have a bearing on the outcome of the test. Following the observations published by Cox & Olitsky (9), it has been customary to incubate the serum-virus mixtures at 37°C., for 2 hr. in a standard test (3) in which albino Swiss mice are inoculated intracerebrally with the mixtures. However, Labzoffsky (10) reported that by prolonging the incubation period to 24 hr. at 37°C., a higher degree of neutralization resulted. This was confirmed by neutralization tests in Colorado tick fever infections (11). Conversely, the results of Crawley (12) and of Olitsky & Casals (13) seemed to indicate that the incubation period may be omitted from the procedure when dealing with the viruses of eastern equine encephalomyelitis (12, 13), western equine encephalomyelitis, St. Louis encephalitis, Japanese encephalitis, Russian Spring-Summer encephalitis and West Nile encephalitis (13).

When the neutralization test is performed in mice, still other factors may influence the outcome of the results, i.e., the age of the test animals and the route of inoculation employed. Olitsky & Harford (14) found that the neutralization test was highly sensitive when the serum-virus mixtures were introduced by the parenteral instead of the intraneural route. It should be pointed out that the determination of age of the test mice to be used must be based on whether they are still highly susceptible to a given virus injected parenterally (15). For example, in case of Venezuelan equine encephalomyelitis (16) and Russian Spring-Summer encephalitis viruses (13), mice of any age may be employed. In other encephalitides, such as West Nile, St. Louis, and Japanese encephalitis, mice between three and fourteen days of age have to be used in order to satisfy the requirements of the test (16). This "parenteral" type of test indicated a high degree of neutralizing power of sera from humans vaccinated against western equine encephalomyelitis (17), while a parallel intracerebral test gave equivocal or negative results. Meiklejohn *et al.* (18) in a comparative study of the intracerebral and intraperitoneal types of neutralization tests, as well as of the complement fixation test, found that the intraperitoneal type of test showed the presence of antibodies against St. Louis encephalitis in cases in which the intracerebral test gave negative results.

Although mice seem to remain the animals of choice for the neutralization test in viral encephalitides, attempts have been made in the past (19 to 23)

to employ fertile hens' eggs for the purpose, taking as the criterion of neutralization prevention of death of the embryo by the virus when mixed with immune serum. Comparison of the egg and mouse tests in Venezuelan equine encephalomyelitis (23) indicated that the sensitivity of the former is equal to that of the intracerebral mouse neutralization test, but much lower than that of the intraperitoneal test. However, Crawley (12), in a recent well-controlled study, found that if the eastern equine encephalomyelitis virus is inoculated together with immune serum into the yolk sacs of 15-day old embryonated eggs, the test is quite sensitive for detection of antibodies but this is not the case when the mixture is injected on the chorioallantoic membranes.

When the neutralization tests are performed with reasonable care and accuracy, the results are usually clear cut. In some instances, however, neutralization has been observed in the sera of hosts which apparently had not been exposed to that particular virus. For instance, sera of certain neotropic marsupials and rodents were found to inactivate the viruses of West Nile, Japanese encephalitis and St. Louis encephalitis, in addition to the yellow fever virus, and yet exposure could have occurred only to the latter (24). The observations of Kasahara *et al.* (25) and Sabin (26) also seem to indicate the existence in some human sera of nonspecific substances which neutralize Japanese encephalitis virus. These reports are of interest in view of the findings of Casals & Olitsky (27) that a lipoid fraction of normal serum may inactivate viruses of certain encephalitides, and of the observation of Utz (28) that lecithin-like and cephalin-like fractions of certain animal sera inactivate psittacosis virus.

The neutralization test has been used extensively by many workers for conducting immunity surveys and for the identification of viruses. It is impossible to cite all the references, but it should be mentioned that the test has been instrumental in identifying the encephalomyocarditis—Mengo encephalitis—MM—and Columbia SK viruses as strains of a single virus (29, 30, 31). Also, Warren *et al.* (32) found neutralizing antibodies against encephalomyocarditis virus in the sera of wild rats trapped in various parts of the United States, the incidence of positives reaching 87 per cent in animals caught in the State of Mississippi.

The neutralization test has been of particular value for the detection of antibodies in sera of persons vaccinated against Japanese encephalitis. Warren *et al.* (33) in a group of vaccinees found complement-fixing antibodies in only one instance as compared to neutralizing antibodies in 30 per cent. The presence of neutralizing antibodies against Japanese encephalitis virus in the sera of "normal" persons in Korea indicated a very high incidence of unapparent infections (34). Salafranca & Espiritu (35) found neutralizing antibodies in the sera of humans and of animals in the Philippines, while Deuel *et al.* (34) in Korea and Burns *et al.* (36) in Japan were able to detect specific neutralizing antibodies in the sera of some domestic animals, with a particularly high incidence among pigs (34).

*Coxsackie virus group.*—A neutralization test in which serial dilutions of

human convalescent sera were mixed with one dilution of virus and then injected intracerebrally into suckling mice was employed by Dalldorf *et al.* (37, 38) to determine the nature of the two agents isolated from fecal material obtained from two clinical cases in Coxsackie, N.Y. Although this type of test was found to be more sensitive (39) than the one in which serial dilutions of virus are mixed with undiluted serum, Melnick *et al.* (40) and Curnen *et al.* (41) employed the latter type of test and nonetheless were able to identify five strains of Coxsackie viruses isolated from cases of so-called nonparalytic poliomyelitis, aseptic meningitis or "summer grippe" (42). Cross neutralization tests, in which specific antisera from hyperimmunized animals (mouse, hamster, monkey, rabbit) were employed, indicated the existence of at least six immunologically unrelated viruses belonging to the Coxsackie virus group (39, 43).

*Poliomyelitis.*—In the study of poliomyelitis viruses, the neutralization test is of primary importance since this test, and the cross-resistance test (see below) are the only ones which are recognized as valid tools in the field of serological investigations of poliomyelitis.[1] Either monkeys or mice are used, depending upon whether monkey-adapted or rodent-adapted strains of poliomyelitis virus are employed. The experience of various workers with the neutralization test in monkeys has been reviewed by Schaeffer & Muckenfuss (45). Recently Steigman & Sabin (46) described a series of neutralization tests in monkeys in which nine strains recovered from human cases were run in parallel tests with acute and convalescent phase homologous sera. When undiluted sera were tested against serial dilutions of virus, both the acute and the convalescent phase specimens showed equal amounts of neutralizing antibodies. However, when serial dilutions of sera were tested against a single dose of virus, the antibody titer was found to rise progressively over a period of weeks during the convalescence. Thus, the results indicated again the greater sensitivity of the technique in which serial dilutions are tested against a single dose ($\pm 50$ $PD_{50}$) of virus. It may be of interest to note that though the viruses recovered from the nine patients were unrelated to the Lansing strain, antibodies against the latter strain were detected in two of the patients in serum specimens obtained three months after onset (no Lansing antibodies were present in the homologous acute phase sera).

The rodent-adapted strains of poliomyelitis have been employed extensively in the mouse neutralization test. Morgan (47) recently has set forth a standard technique and Bell (48) has studied the variables. Results of both workers indicate that reproducibility and sensitivity of the test are greatly enhanced if serial dilutions of serum are tested in the presence of a constant amount of virus. Employing this method, Morgan (49) was able to demonstrate the presence of specific antiviral substances in the areas of the central nervous system of paralyzed monkeys infected with the Lansing strain.

[1] The flocculation test described by Roberts (44) needs further confirmation before it can be accepted as a valuable tool in poliomyelitis research.

These results paralleled those observed by Gard (50) in mice, paralyzed as a result of infection with Theiler's mouse encephalomyelitis virus. Conversely, Sabin & Steigman (51), working with a strain of poliomyelitis virus not related to the Lansing strain, failed to detect the presence of homologous antiviral substances in the central nervous system of paralyzed monkeys. Whether the discrepancy in the results obtained can be explained by the difference in strains employed remains to be seen. This problem seems to be of paramount importance in the evaluation of the part played by humoral antibodies in relation to the actual resistance to infection. In connection with the above, it has been reported recently (52) that antiviral substances against poliomyelitis virus are present in nasopharyngeal secretions (53, 54).

Finally, an interesting parallel has been drawn by Hammon (55) between the time of appearance and the level of neutralizing antibodies against the Lansing virus in sera of normal children, and the appearance and level of antistreptolysin "O" in sera of the same individuals. A very close relationship was observed, indicating a possible common epidemiological pattern, but having no bearing upon the etiology of the disease. However, this observation may be of value in future epidemiological survey studies of poliomyelitis.

*Dengue.*—Adaptation of the dengue virus (56, 57, 58) to mice made possible the development of a neutralization test (2, 4) in which serial dilutions of virus are mixed with undiluted test serum and inoculated into mice intracerebrally. Because there are at least three different strains (59) and thus far only one of these has been mouse-adapted for use in the neutralization test (4), the absence of neutralizing antibodies does not necessarily indicate that an infection had not taken place in the past with one of the other strains which have not yet been adapted to the mouse.

*Foot-and-mouth disease.*—The method of titrating the virus by inoculation in multiple sites on a bovine tongue (60) permitted the development of an accurate and standardized neutralization test (61) in which serial dilutions of serum in the presence of a constant amount of virus derived from infected bovine epithelium, are inoculated simultaneously into five sites on the tongue, using two or more calves for each test. The neutralization titer is calculated on the basis of the number of lesions produced on the tongue 20 to 28 hr. after inoculation. This test was employed successfully for the identification of virus strains recovered from the recent outbreaks in Mexico (62).

*Mumps.*—The neutralization test is performed in fertile hens' eggs by using the serum dilution technique with a constant amount of virus (4). A detailed description of such a test may be found in an article by Leymaster & Ward (63). The same authors (64) observed that, as in viral encephalitides (6, 7), the neutralizing capacity of immune serum may be reduced by heat inactivation and adsorption with an antigen-antibody precipitate. Reactivation of neutralizing antibodies can be obtained by addition of fresh normal serum. A labile component present in the sera of humans, guinea pigs, and rabbits was found to neutralize mumps virus (65) in the presence of calcium

salts. The combination was stable and did not undergo spontaneous dissociation. This labile component was described as of protein nature but distinct from complement.

*Psittacosis.*—There are two types of neutralization tests described in the literature. In the toxin-antitoxin neutralization test the lethal effect of psittacosis virus toxin is prevented by the use of homologous antitoxin prepared in rabbits or chickens (66). Because of its nature, this test can be applied only for the identification of an unknown toxin-producing pathogen suspected of belonging to the psittacosis-group of viruses. The second test is the viral neutralization test (67, 68) in which potent antisera are obtained by the hyperimmunization of roosters. The use of this latter test has helped to establish the immunological identity of several strains of psittacosis virus (68) and to indicate the serological relationship between members of the same group.

*Influenza.*—The hemagglutination-inhibition test has, in the recent past, supplanted the neutralization test. In spite of indications (69, 70) that the hemagglutination-inhibiting antibodies and the neutralizing antibodies are closely related, if not identical, some workers (71, 72) have maintained that the two types of antibodies are dissimilar. Recently, Walker & Horsfall (73) produced evidence for the lack of identity of the hemagglutination-inhibiting antibodies with the neutralizing antibodies. Thus, the neutralization test, performed either in embryonated eggs (73) or in mice (74), may still prove itself of value in immunological investigations of influenza.

In this review only those recent studies have been singled out which provide new information concerning the neutralization test. Summarizing, one may point out that the neutralization test in viral diseases holds its own as an immunological tool. A disadvantage of the test lies in the fact that suspensions containing living pathogens are in constant manipulation during the performance of the test, with attendant danger of exposure to the working personnel. Conversely, because of the greater skill required of the technical help, the execution of the test and evaluation of the results are no doubt carried out with greater care than is true of certain other procedures. Moreover, nonspecific reactions are less likely to occur with the neutralization test, if correctly performed, than with other techniques.

## The Cross-Resistance (Cross-Immunity) Test

*Concept.*—The cross-resistance test is based upon the reaction of a host, (*a*) known to be immune to a given viral disease and inoculated with infectious material presumed to contain a virus of unknown identity, or (*b*) recovered from an unknown infection and challenged by a known viral agent.

*Applicability.*—(*a*) Diagnostically, the test may be employed for the identification of a viral agent causing the disease and (*b*) for the study of the relationship between different viruses.

*Technique.*—As outlined above, the technique involves three procedures: (*a*) artificial immunization of a host by means of inoculation with inactivated vaccine or exposure to living virus, and use of the host after recovery;

(*b*) verification of immunity, if feasible, through artificial exposure to homologous virus and (*c*) exposure to a heterologous virus and careful determination of any reactions in the "immune host" as compared to a non-immune host (control) of the same species.

In descriptions of immunological procedures, the cross-resistance test is not actually ignored but neither is it particularly stressed, presumably because the scope is much narrower than of the other immunological techniques. Yet this test is invaluable in those viral diseases in which no *in vitro* test is available and in which the neutralization test is impossible or difficult to perform because of technical reasons. Therefore, the following discussion of the cross-resistance test will be limited to those instances in which it has contributed materially to greater knowledge of a given viral disease.

*Common cold.*—Although the first successful transmission and immunological studies in this disease were performed in chimpanzees (75) the cost and unavailability of these apes precluded any large scale experiments. Research workers, therefore, turned to human volunteers in an attempt to investigate the common cold entity. A considerable amount of work has been performed in the recent past by the Commission on Acute Respiratory Diseases of the U.S. Army. Two agents have been isolated which cause clinically distinct entities: The acute respiratory disease (ARD), characterized by sore throat and a long incubation period (76), and the severe common cold (SCC), characterized by coryza and short incubation period (76). The two filterable agents were then used in cross-resistance tests in human volunteers, with the following results. Volunteers recovered from the induced ARD-infection were resistant to reinoculation with homologous virus, but not to induced SCC-infection. On the other hand, volunteers recovered from induced SCC-infection were resistant neither to the homologous (SCC) reinfection nor to the heterologous (ARD) challenge. Neither of the two agents induced resistance to infection with an agent derived from cases of primary atypical pneumonia (77). On the other hand, Pollard & Caplovitz (78) found that six out of eight individuals who had been successfully infected with the common cold agent, derived from nasal washings of individuals sick with acute common cold infection, were refractory to a reinoculation with the same material 11 to 13 days later. These same authors have reported that they have adapted their strains of common cold virus to embryonated eggs, basing their claim on the observation that material obtained from the ninth egg-passage was able to "immunize" volunteers against a challenge inoculation given one week after the second immunizing injection. However, numerous attempts (79, 80) by British workers to cultivate the cold virus in fertile hens' eggs have not only ended in failure, but their work indicated that normal embryonic membranes and fluids may provoke symptoms similar to those of a common cold. This evidence, stemming as it does from workers who have had considerable experience in studies with human volunteers (81, 82) should not be disregarded in future evaluations of immunological studies. If the "challenge" inoculation with the common cold is to be introduced through the same route as the "immunizing" agent, then only

extremely well controlled and statistically large studies will lead to reliable conclusions regarding immunological relationships within the group of com mon cold viruses.

*Infectious hepatitis and homologous serum jaundice.*—In spite of numerous attempts to transmit the causative viruses of these diseases to experimental animals, only in a few instances have any reactions, classified as equivocal (83), been evoked (84, 85, 86). Even when the "infectious" material was passaged serially in a large variety of hosts, the experiments invariably ended in failure. Also, attempts to elaborate an immunological test *in vitro* have not passed much beyond the preliminary stages (87, 88). Thus, the only susceptible host available for the study of these viruses is the human volunteer. The results of cross-resistance tests may be summarized as follows: Humans convalescing from experimentally induced homologous serum jaundice are resistant to a second inoculation of homologous virus (89). However, they are fully susceptible to infection with the virus of infectious hepatitis (89, 90). Immunity studies in volunteers have confirmed epidemiologic observations that patients who have recovered from either infectious hepatitis or homologous serum jaundice are susceptible when exposed to the other disease (91). Only the results of Oliphant (92) indicated that homologous serum jaundice infection may induce resistance to infectious hepatitis, but it has been shown that this strain of infectious hepatitis virus has many traits characteristic of the homologous serum jaundice virus (93).

*Dengue and phlebotomus fever.*—Cross-immunity studies in human volunteers, supplemented by neutralization tests with the single mouse-adapted strain (see above), have demonstrated the existence of three distinct immunological types of dengue virus (59). However, some degree of relationship was indicated by a state of resistance in human volunteers who recovered from one type of infection to reinfection by a heterologous type. The period of complete resistance lasted two months, and partial resistance eight months (94). Human volunteers recovered from dengue were found susceptible to infection with Colorado tick fever (95), indicating lack of immunological relationship between the two diseases.

No other vertebrate host than man has been found to be susceptible to infection with phlebotomus fever (94). Cross-immunity studies performed with volunteers have indicated the existence of at least two immunological types: one strain, recovered from an outbreak in Naples, was distinct from two other strains recovered from outbreaks in Sicily and the Middle East; the latter two were immunologically identical (59).

*Foot-and-mouth disease.*—In foot-and-mouth disease both the neutralization test (see above) and the complement-fixation test are currently employed. Yet the cross-resistance test, in which animals were inoculated with inactive vaccine and then challenged with either homologous or heterologous strains of virus, has revealed more subtle antigenic differences than the accepted main immunological groups of O, A, and C (96). The same type of test, employed in investigations of the Mexican foot-and-mouth outbreak, indicated that though the Mexican strains were classifiable in the A group,

vaccines prepared from a classical 119 A type strain failed to protect the cattle against infection with the Mexican strain (97). These observations may justify Verge's (98) statement that the cross-resistance test in guinea pigs and cattle should be given preference over the complement-fixation test in the identification of newly isolated strains.

*The pox viruses.*—The relationship between vaccinia and mousepox (infectious ectromelia) viruses was shown to exist by the cross-resistance test in mice (99). Studies on the protection of mice against virulent mousepox infection by immunization with vaccinia virus further revealed (100) that although some multiplication of mousepox virus occurs in the vaccinia-immunized mice, the virus is confined almost entirely to the local lesion and only very rarely does a secondary viremia and skin rash occur. Ultraviolet-irradiated vaccinia protected the mice against challenge with mousepox virus (101) but failed to immunize rabbits against a challenge with vaccinia virus. An interesting observation on the mechanism of immunity induced by ultraviolet-irradiated mousepox virus vaccines was made by Andrewes & Elford (102), who found that if small amounts of active virus are mixed with large amounts of inactive virus, the activity of the former often will be markedly enhanced and solid immunity will be induced. This raises the point whether a sudden deterioration of potency in ultraviolet-irradiated viral vaccines may not be due to the "dying" of small amounts of living virus originally present. Cross-resistance tests in monkeys indicated that six strains of alastrim virus (five African and one originating in the United States) are related immunologically (103), although minor qualitative antigenic differences were noted.

*Poliomyelitis.*—Use of the cross-resistance test in rhesus monkeys has shed considerable light on strain differences. Two types of cross-resistance tests have been employed for this purpose. In one type, monkeys were subjected to repeated parenteral inoculations with a specific strain of living virus, challenged by intracerebral route with the same strain and, if symptomless, were given one more parenteral injection of the homologous virus. These animals were then divided in groups and challenged with large intracerebral doses of unknown strains (104, 105, 106). In the second type of test, rhesus monkeys convalescing from an experimentally induced attack with a known strain of virus were challenged by the intracerebral route with unknown strains (107, 108). Both techniques gave essentially the same results in grouping the various strains of poliomyelitis virus into three immunological entities: the Brunhilde, Lansing, and the Leon types. However, studies conducted by Bodian (108) and by Morgan (109) indicate that the relationship between these groups of viruses may be of more subtle nature than originally determined. Bodian (108), using the Lansing and Brunhilde strains, found that paralytic convalescent monkeys, which were solidly immune to an intracerebral challenge with a homologous strain, were also partially resistant to a heterologous strain. This indicated that some relationship between these two distinct groups of viruses may exist. Morgan (109), studying the appearance of neutralizing antibodies in the central

nervous system of monkeys vaccinated parenterally with the Lansing strain, observed that intracerebral challenge with an unrelated (Minnesota) strain gave rise to antibodies to the Lansing strain. The level of the anti-Lansing serum antibodies in the blood of parenterally vaccinated monkeys correlated with the resistance to intracerebral challenge with the same virus, and the minimum protective titer of serum in such animals was found to be 1:1000 (110).

*Coxsackie virus group.*—Since only suckling mice are fully susceptible to this infection, a method had to be devised to transfer immunity to the baby mice. This was done by immunizing the pregnant mice and then the infant mice were challenged with several strains of the virus (39). The results of this ingenious cross-resistance test seem to confirm those of the neutralization test (see above) on the immunological multiplicity of the virus strains (39).

*Psittacosis and infectious bronchitis.*—In a study based on cross-resistance tests in mice, Wagner *et al.* (111) came to the conclusion that the strains of avian origin possess a broader antigenic pattern than do the strains of mammalian origin in the psittacosis-lymphogranuloma venereum group of viruses.

The three strains of infectious bronchitis isolated in England and submitted to cross-resistance tests in chickens by Asplin (112) were found to be identical. Moreover, one of the strains was immunologically identical with an American strain.

*Viral encephalitides—local (cerebral) versus humoral immunity.*—In the section on neutralization tests in poliomyelitis, passing mention was made of the controversy regarding the presence or absence of antiviral substances in the brains of monkeys paralyzed after intracerebral inoculations with poliomyelitis virus. The problem of "local" immunity in mice immunized against western equine encephalomyelitis and challenged intracerebrally with homologous virus has been the subject of study by Schlesinger (113, 114). The reader is referred to the original papers for detailed results, but Schlesinger found that the mechanism of resistance of the vaccinated animals is closely related to the local (central nervous system) concentration of virus neutralizing antibodies. The problem of cellular resistance to viral infections has been reviewed recently by Enders (115).

In closing the section on cross-resistance tests, a brief note should be made of a possible complicating factor in the procedure. If immunization of either humans or animals is done with living virus and then the challenge material is introduced by the same route, one may encounter the interference phenomenon rather than immunity. It is good practice to use different routes for the immunizing injections and the challenge inoculations. Also, immunization with inactive virus, in some instances, may be preferable. In connection with the latter procedure, mention may be made of two instances in which the results of challenge with the same viral preparation seemed to depend on the tissue menstruum used for the preparation of the vaccines. In the cases of Japanese encephalitis (116) and louping-ill (117) vaccines prepared

from homologous type of tissue seemed to give better protection to the vaccinated animals than did the use of heterologous type of tissue. Whether these observations represent only isolated instances or more commonly existing phenomena further research should disclose.

### The Hemagglutination-Inhibition Test

*Concept.*—This test is based upon the ability of immune sera to interfere with the agglutination of erythrocytes by certain viruses.

*Applicability.*—(*a*) For diagnostic purposes, for the detection of antibodies in the sera of persons or animals sick or convalescing from a suspected viral disease, and (*b*) for the study of relationships between those viruses which are able to cause hemagglutination.

*Technique.*—Usually a constant amount of virus is used and mixed with serial twofold dilutions of serum. Erythrocytes, in 1.0 to 0.25 per cent concentration, are added and the test is allowed to incubate at room temperature for 1 to 1½ hr., when it is read for the inhibiting titer of the serum. There are many detailed descriptions of the test (1, 2, 3, 4) and a very useful type, known as the pattern test, has been reported by Salk (118). Recently a method has been described by Himmelweit (119) which yields reliable results and enables titration of virus hemagglutination, as well as the determination of agglutination-inhibiting antibodies with small amounts of material.

The following viruses have been reported to produce hemagglutination and can be used, theoretically at least, in the hemagglutination-inhibition test: Influenza A and B, swine influenza, smallpox, vaccinia, mousepox, fowl plague, Newcastle disease, penumonia virus of mice (3), and in addition recently the Columbia SK—MM—encephalomyocarditis—Mengo group (120 to 123) and the GD VII strain of Theiler's mouse encephalomyelitis virus (124). Hemagglutination of rat red blood cells with foot-and-mouth disease virus has been described (125) but this report requires further confirmation. The biological implications of hemagglutination by viruses were discussed by Jawetz (126).

Before considering the hemagglutination-inhibiting test in relation to individual viral diseases, it is appropriate to mention that there are several substances unrelated to specific antibodies which will also inhibit, or certainly modify, the hemagglutination properties of viruses (127). In normal animal or human sera two types of inhibitors have been described: one a thermolabile component of possible protein nature (128), and the other a thermostable substance of probable mucoid nature (129) which bears a resemblance to the inhibitors isolated from human ovarian cysts (130), and egg white (131). Allantoic fluid (132), extracts of many organs (133), and extracts of red cells (134) also contain agglutination inhibitors. The inhibitor substance present in human serum has been partially purified by chemical fractionation (135) and that present in egg white by precipitation in an electrical field (136). It has been observed that the mucoid type of inhibitors can be destroyed with large amounts of periodate (127). The union of the inhibitor and virus may be reversible (127), and the influenza virus in pres-

ence of inhibitor from the allantoic fluid is as infectious as the control virus (137). Living virus can inactivate the inhibitor (138).

*Mumps.*—A modification of the agglutination-inhibition test, introduced by Robbins *et al.* (139), consists of the use of larger amounts of virus-hemagglutinins, prolongation of the incubation period of serum-virus mixtures prior to the addition of erythrocytes, and reading of the results of the tests before complete settling of the erythrocytes. This procedure has permitted an adequate distinction between the titers of acute and convalescent phase sera in the diagnosis of mumps in cases of parotitis and meningoencephalitis without parotitis. The results of the test were further substantiated by complement-fixation tests and by the isolation of the virus.

Conversely, the results of Kilham *et al.* (140) seem to indicate that diagnosis of mumps or Newcastle disease based only upon the presence of antiagglutinins should be made with caution since of the 22 sera of mumps-convalescent patients tested, 11 showed anti-agglutinins which inhibited also the Newcastle disease virus. Because of the prevalence of the latter virus among fowl and possible exposure of humans, the mere presence of anti-hemagglutinins may not suffice to distinguish between the two diseases. Virus isolation and neutralization tests are also indicated.

Mention should be made of another sensitive type of test used for the detection of mumps antibody in human serum (141). This is based on the reaction of serum with erythrocytes previously coated with a virus antigen.

*Infectious mononucleosis.*—The original observation on the agglutinability of erythrocytes coated with Newcastle disease virus by sera of patients with infectious mononucleosis (142) has been extended by other workers. These agglutinins are distinct from the specific Newcastle virus agglutination-inhibiting antibodies (143, 144), but are found only in a certain percentage of sera of humans suffering from infectious mononucleosis. Sera of some patients suffering from diseases other than infectious mononucleosis were found to show higher agglutinative titers for Newcastle virus-coated erythrocytes than normal sera (143). Included in this group were two cases of infectious hepatitis and it may be of interest to note that Bang (145) found a factor in old hepatitis sera capable of agglutinating normal chicken erythrocytes.

*Influenza.*—An investigation (146) on the validity of the virus hemagglutination reaction for determining the titer of antibodies in sera indicated that the use of heat-killed virus is likely to yield more uniform results provided that the normal serum inhibitor (see above) is completely removed. The authors seem to have accomplished the latter by treating the sera with an enzyme present in *Vibrio cholerae* filtrates. Another technique of antibody titration was introduced by Fazekas de St. Groth (147) who used erythrocytes treated with periodate and then coated with influenza virus. These cells, from which the *Vibrio chloerae* enzyme cannot elute the virus, are stable in suspension and have the virus bound firmly to the modified receptors; they are used for the determination of antibody content. Substitution of slides for tubes in the test has been proposed by Bakos & Nordberg (148).

The use of hamster immune sera in the hemagglutination-inhibition test revealed small but significant differences between strains isolated from the same institutional outbreak of influenza (149).

The hemagglutination-inhibition test, when it was applied to three strains of type B influenza virus isolated in Seattle, indicated that they deviated slightly in their immunological relation to the standard Lee strain (150). A strain of swine influenza virus isolated in 1939 was found on the basis of the hemagglutination-inhibition test to resemble some members of the human A′ group (151). Although normal egg yolk does not possess any hemagglutination-inhibiting capacity, the yolks of eggs from influenza-immune chickens were found to demonstrate considerable inhibitory power (152).

The bulk of papers published on influenza virus in the recent past deal to a greater extent with the description and nature of the inhibitors (see above) than with the techniques of the test. One should, however, stress once more the need for critical evaluation of the identity (69), or lack of identity (73), between the hemagglutination-inhibiting and neutralizing antibodies.

*Newcastle disease virus.*—The various methods employed for diagnostic purposes were summarized by Brandly *et al.* (153). The application of the hemagglutination-inhibition test has been studied by Beach (154), who observed that sera which had the same virus-neutralizing titer differed markedly in the hemagglutination-inhibiting titers. These studies seem to indicate again a possible lack of identity between the two types of antibodies.

An improved type of test was reported by Fabricant (155), who advised the use of untreated, rather than formalinized, allantoic fluid for source of virus and incubation of the test at 25°C., rather than at room temperature. The author also proposed to consider a serum titer of 1:160, or higher, as positive for diagnosis of Newcastle disease.

*Neurotropic viruses.*—The report (120, 121) that the Columbia SK—MM—Mengo—encephalomyocarditis group of viruses caused agglutination of sheep erythrocytes prompted further research in this field. The results of Lahelle & Horsfall (124) indicated that the GD VII strain of mouse encephalomyelitis virus caused agglutination of human group O erythrocytes at 4°C. Sera containing antibodies either against the GD VII strain or FA strain of mouse encephalomyelitis specifically inhibited the hemagglutination reaction, yet neither the FA strain nor the Lansing, MEF 1 and Brunhilde strains of poliomyelitis virus were found to cause an agglutination reaction. The Columbia SK—MM—Mengo—encephalomyocarditis viruses caused agglutination of sheep erythrocytes (122) and again group specific antiserum inhibited the reaction. Seventeen other neurotropic viruses (122), including certain members of the Coxsackie group (156), failed to agglutinate erythrocytes derived from eight animal species. Thus a simple *in vitro* test for the diagnosis of neurotropic viral infections still remains applicable to only two groups of viruses.

*The pox viruses.*—Buffalo dermal pulp infected with vaccinia virus was found to be an excellent source of hemagglutinins even after 18 years' storage

in glycerol (157). The hemagglutinating factor seemed to be closely related to the S-antigen; it is thermostable and formalin-resistant. In the majority of cases the hemagglutination-inhibiting titers in sera of humans rise after vaccination against smallpox (158). An antigenic relationship between mousepox and vaccinia virus was demonstrated by complement-fixation and hemagglutination tests (159).

## The Complement-Fixation Test

*Concept.*—This test is based upon the binding of the complement by a specific antigen-antibody combination, making the former unavailable for lysis of sensitized erythrocytes.

*Applicability.*—(*a*) For diagnostic purposes (i) to determine the presence or absence of antibodies in a serum against a given virus, and (ii) to identify a virus, provided the latter, when passaged in laboratory animals or eggs, can be used as complement-fixing antigen; (*b*) for the determination of the potency of antiviral sera, and (*c*) for the study of cross-relationships of different viruses.

*Technique.*—The technique of the test has been extensively described (1, 2, 3). In addition, a very good review of the currently employed procedures may be found in the publication by Kabat & Mayer (160). Discussion of certain fundamental concepts in the complement-fixation test and a proposed uniform notation have been presented by Thompson *et al.* (161). A technical innovation in the test, in which drops on Perspex (plastic) sheets replace larger volumes of reagents in tubes, has been introduced by Fulton & Dumbell (162) for use in the study of influenza virus, and has been employed successfully in studies on Coxsackie virus (39).

*Viral encephalitides.*—The use of infected mouse brain as a source of complement-fixing antigen had certain disadvantages, since it reacted nonspecifically with Wassermann positive sera. Several methods have been devised to render the antigens more specific without affecting their original potency. Benzene extraction was introduced by DeBoer & Cox (163) and certain modifications in the technique were suggested by Espana & Hammon (164). Recently an acetone-ether extraction procedure has been described (165) which yields non-anticomplementary and specific antigens within 6 hr. from the time the infected tissue is harvested. The same technique was applied successfully in preparing antigens from tissues infected with Coxsackie viruses (156).

Benzene-extracted antigens, centrifuged at 13,000 r.p.m. for 1 hr. to remove all the anticomplementary properties, have been used in dengue fever (166). Employing "most potent preparations," Sabin (167) demonstrated an antigenic relationship between dengue, yellow fever, West Nile, and Japanese encephalitis. A new method of preparing complement-fixing antigens was recently described by Warren *et al.* (168) who purified Japanese encephalitis virus by (*a*) precipitation of the nonspecific proteins with protamine, (*b*) removal of the excess of protamine from the supernate by precipitation with heparin, and (*c*) concentration of the active principle by

ultracentrifugation. Such highly purified preparations gave good results when used as complement-fixing antigens. A similar method was used successfully for preparing antigen for infections with Coxsackie viruses (39).

*Mumps.*—The nature of the complement-fixing antigen was studied by Lind (169) who found a close relationship between the latter and the hemagglutinin and the erythrocyte-sensitizing agent. All three activities appear to be properties of the virus particles themselves. Pursuing further their studies on Viral (V) and Soluble (S) antigens in mumps, Henle *et al.* (170) found that antibodies against S-antigen may be observed earlier in the disease and reach high levels before the antibodies against V-antigen appear. Later in convalescence, the levels of anti-S antibodies decrease and anti-V increase. After vaccination (or skin testing), although both types of antibodies may develop, the anti-V increases more regularly and to higher titers. The value of the complement-fixation test for the diagnosis of mumps was further confirmed by Rice & Walker (171) and by Oldfelt (172). A comparison of the diagnostic value of the complement-fixation test with the hemagglutination-inhibition test (173, 174) indicated that the former seems to give more reliable and more reproducible results. The relative values of the complement-fixation test, the agglutination-inhibition, and the modified human erythrocyte-agglutination tests (141) were compared for the laboratory diagnosis of mumps (175). Antibody rise was demonstrated early in the course of illness by all three methods, but higher titers were obtained when the human erythrocyte-agglutination test was employed. Nonconcentrated infected allantoic fluids were found to be as satisfactory a source of complement-fixing antigens as were the "viral" antigens prepared from concentrated allantoic fluids, or "soluble" antigens obtained by concentrating chorioallantoic membranes (175). Rice (176) found higher antibody titers during the acute phase of illness when infected chorioallantoic membranes, instead of allantoic fluids, were used as source of antigen.

*Influenza.*—A study (177) of the infectivity and of the hemagglutinating and complement-fixing antibodies during the first infectious cycle (in chick embryos) of influenza A virus indicated that though the infectivity remains constant for 5 to 6 hr. after inoculation, the titers of hemagglutinins and complement-fixing antigens in the allantoic fluids begin to rise 3 hr. after inoculation. The soluble and viral antigens can be demonstrated in allantoic membranes as early as 2 hr. after incubation and 2 hr. prior to rise of infectivity. Antisera for use in the complement-fixation test were prepared in hamsters by intranasal instillation of infected allantoic fluids and several strains and types of viruses were studied, employing either infected allantoic fluids or allantoic membranes as antigens (178).

*Foot-and-mouth disease.*—Infected bovine epithelium, first purified by centrifugation and then precipitated with ammonium sulphate, was used as antigen (179, 180). Convalescent guinea pig sera were used after inactivation at 56°C., and bovine sera were inactivated at 60°C. for 20 min. (180) to eliminate nonspecific effects. With the complement-fixation test, the two strains of type A recovered from the outbreak in Mexico were found to dif-

fer antigenically from each other as well as from the standard strain 119 of type A (181).

*Psittacosis-lymphogranuloma venereum group of viruses.*—Although complement-fixation and agglutination tests are the most commonly employed immunological tests (2, 4), considerable cross reactions are known to occur between different members of this group of viruses in the complement-fixation test. Satisfactory complement-fixing antigens for psittacosis were prepared from infected allantoic fluids phenolized and kept for several days at 4°C. The active principle was further concentrated by centrifugation. It was found that fresh yolk or lecithin may increase the potency of the antigen (182). The complement-fixation test was used for diagnostic purposes in a study of 21 cases in humans; the test became positive about the seventh or tenth day after onset, reached a maximum titer about the 15th to 30th day and persisted (in low titer) for a considerable length of time (183).

Yolk sacs infected with lymphogranuloma venereum, meningopneumonitis or mouse pneumonitis antigens were fractionated with organic solvents (184) and active complement-fixing fractions could be extracted with either ether or chloroform. Fractions insoluble in alcohol-acetone, devoid per se of activity, were activated by the addition of optimal amounts of lecithin. Both methods yielded a purified, highly active group-specific antigen (184). Yolk sacs, infected with either psittacosis or lymphogranuloma venereum viruses, extracted with 0.02 *N* hydrochloric acid at 37°C., yielded a specific antigen which gave positive results when employed in skin testing of patients, but gave negative results when used in the complement-fixation test (185).

*Indirect complement-fixation test.*—Working with sera obtained from certain avian species immunized against *Salmonella pullorum*, Rice (186) failed to obtain positive complement fixation. However, she observed at the same time that such avian sera when added to rabbit or guinea pig sera containing antibodies against *S. pullorum* inhibited the fixation of complement by the latter sera in the presence of *S. pullorum* antigen. These observations led to the technique of the "indirect" complement-fixation test (187) which, first applied to *S. pullorum* of turkeys (188), was extended by Wolfe *et al.* (189) to Newcastle disease of chickens. The latter authors, who simplified the technique of the test, found very good correlation between specific antibodies measured by means of the indirect complement-fixation test and the hemagglutination-inhibition test. Hilleman & Helmold (190) applied the indirect complement-fixation test to the study of antibodies against the psittacosis-lymphogranuloma venereum group of viruses in sera of chickens and observed that the results indicate a group specificity similar to that displayed by mammalian sera in the complement-fixation test, rather than the strain specificity found in chicken antisera by the neutralization test (67).

## Remarks

As mentioned previously, the literature pertinent to the subject of this survey is far too extensive to be cited in full. In the selection of articles, the

reviewer has tried to avoid arbitrary judgment and to select those of greatest general interest. If some publications, whose authors consider their contributions to the subject under review as being important, have failed to be mentioned, the authors may be assured that the omissions were not intentional but dictated chiefly by the limitations of space.


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# THE IMMUNOLOGY OF THE HUMAN MYCOSES[1,2]

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## Introduction

The immunological aspect of medical mycology has tended to lag behind similar aspects of other fields. It appears, however, that immunologic processes in the fungus-infected host are essentially similar to those which occur in bacterial, viral, and other diseases of infectious etiology. The procedures and techniques for measuring immunologic reactions are, therefore, similar to those which have been established for the infectious diseases in general. Possibly because of the somewhat more highly differentiated structure of the fungi, the preparation of antigens for immunologic studies requires certain modifications. Except for this difference no really specialized knowledge appears to be necessary in the performance of immunologic tests.

As in other infectious diseases, immunologic data are of diagnostic and prognostic consequence only when interpreted in the light of the limitations of the commonly used tests. In some instances negative results, such as negative tests for cutaneous sensitivity, are of greater significance in excluding a particular diagnosis than are positive tests in establishing it, as exemplified by the tuberculin test and, similarly, by the coccidioidin test for coccidioidomycosis. Immunologic studies, however, constitute an important part of the clinical evaluation in respect to diagnosis, prognosis, and management of many of the systemic mycoses.

The systemic mycoses are insidiously developing, chronic, and often fatal diseases. They tend to simulate tuberculosis. Consequently, there are many pathologic and immunologic analogies between the mycoses and chronic bacterial diseases.

The pathogenic fungi may, on the one hand, be characterized as being more allergenic than bacteria, in the sense that cutaneous sensitivity is practically a constant feature of the human mycoses. On the other hand, they may be said to be less antigenic than many bacterial pathogens in that concentrations of circulating antibodies, expressed as antibody titers, are generally low. For example, although a positive complement-fixation test with undiluted serum or serum diluted 1:2 might be skeptically regarded in other infections, such low titers may assume diagnostic significance in the human mycoses, as is the case in blastomycosis (1). The reasons for this meager antigenicity are not well known. It is possible that the slow progression

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of the mycoses and the relative scarcity of organisms in the lesions partially account for this phenomenon. However, even following hyperimmunization with large quantities of such organisms as *Cryptococcus neoformans* and *Blastomyces dermatitidis*, the antibody response may be very low.

Because of the low titers, the mechanical details of performing the serologic tests are of considerable importance, and emphasis must also be placed upon the dependent difficulties of interpretation. Much work yet remains to be done upon the standardization of antigens and the establishment of routine methods to yield the most reliable results.

The pathogenic fungi as a group, with the possible exception of *Aspergillus fumigatus* (2), are not known to produce endotoxins or exotoxins. Mycologic immunology, therefore, does not include reactions based on antitoxins.

The fungi which cause human disease may be arbitrarily divided into two distinct groups: (*a*) the superficial or "ringworm" fungi, and (*b*) the systemic fungi. These two groups comprise separate general subjects which will be dealt with individually. The immunology of the superficial mycoses will be very briefly stated; the immunology of the systemic fungi will be dealt with in detail.

## Human Susceptibility to Fungus Infections

Many empiric observations have been made which are of interest in this field, but there has been relatively little experimental work. More is known about susceptibility to the ringworm fungi than to the systemic pathogens. Adults appear to be uniformly susceptible to the organisms which cause ringworm of the foot (athlete's foot). Rothman (31) has explained this in terms of the observed lack or reduction of sebum secretion in the areas attacked by the fungus. Sebum has been demonstrated to contain long-chain saturated fatty acids which are fungistatic (32, 33, 34). Athlete's foot, however, is an uncommon disease among children. It seems unlikely, therefore, that Rothman's hypothesis alone can account for the low incidence of infection in children unless one assumes that there is a great difference in exposure to the source of infection. Ringworm of the scalp, on the other hand, is a common children's disease and a rarity among adults. Rothman and his colleagues explain the immunity of the adult scalp on the basis of an increased amount of fatty acids in the sebum of adults (34). This view has been questioned (35). Should Rothman's view eventually be proved correct, then the natural resistance exhibited by adults would not be strictly an immunologic phenomenon, but rather a physiologic one dependent upon the increased sebum formation which occurs after puberty. However, in a recent series of experimental inoculations of *Microsporum audouini* (the organism causing epidemic tinea capitis), Kligman was unable to produce infection in many children, and the resistance exhibited by the adult appeared to differ only quantitatively, since some adults could be infected with this fungus. It would appear that although there are differences in natural resistance between adults and children to different types of ringworm infection, the cause for these differences at present requires further elucidation, and this cause may have little relationship to the factors concerned in acquired immunity.

So far as the deep mycoses are concerned, serious investigations of the existence of acquired immunity have not been made. In certain of these diseases, circulating antibodies regularly develop in appropriate animals and in humans. The mere presence of such antibodies does not imply an increased capacity on the part of the host to resist or control infection. Although it is quite likely that acquired immunity does develop in certain mycoses, experimental proof is lacking. It is interesting that Henrici (36) thought of the allergic state induced by the systemic fungi as a disadvantage to the host. He postulated that the spread and dissemination of lesions in these diseases resulted from hypersensitivity. In fact, he regarded the deep fungi as basically saprophytes which could acquire the pathogenic habit peculiarly as a result of their capacity to sensitize. Evidence for this view is not at hand.

## Immunology of Ringworm Infections

The ringworm fungi have a limited pathogenicity, proliferating in the dead stratum corneum of the skin and the skin appendages, i.e., the nails and hair. They are consequently of little significance so far as general health is concerned.

These fungi usually do not incite the development of circulating antibodies so that immunologic study is limited in the main to cutaneous tests. They often cause inflammatory disease which is associated with a positive cutaneous sensitivity reaction (3). The antigen or antigens involved are elaborated by all the species of the ringworm group and apparently consist of nitrogen-containing polysaccharides (4 to 7). The cutaneous response to the antigen (trichophytin) is of the delayed tuberculin type, but occasionally the immediate urticarial type of response is observed (8 to 12). The latter reaction has been passively transferred by means of serum to nonsensitive individuals; this, then, is an exception to the rule that circulating antibodies are generally not produced in the superficial mycoses. Agglutinins, precipitins, and complement-fixing antibodies have not been convincingly demonstrated (13, 14).

The basic researches of Bloch, Jadassohn, and many others produced a great deal of immunologic knowledge on experimental ringworm infection in animals (15 to 23). It is not our purpose here to review comprehensively the accumulated studies on immunity and sensitivity in the ringworm infections. The entire field has recently been surveyed and expanded by DeLamater & Benham (24 to 27). Only a general statement of the results will be given. Suffice it to say that experimental infection of an animal with a ringworm fungus (*Trichophyton mentagrophytes*) produces a state of relative acquired immunity to reinfection. The immunity observed is not complete, but is expressed by a shorter course of infection and the relative absence of fungi in the lesions.

During the course of the initial infection a sensitivity to trichophytin is established which is of a more or less persistent nature. The development of this allergy is paralleled by the development of acquired immunity, and many investigators have regarded the allergic state as closely bound to the immunity. According to this view, the acquired immunity is really a special

expression of the allergy. DeLamater (26), however, observed the development of anergy coincident with immunity within the lesion site in experimental ringworm. An apparent similarity with the view that the acquired immunity in tuberculosis is dependent upon the allergic state is suggested, but Rich's (28) researches in tuberculosis have raised issue with this concept. Anderson & Kligman (29) from preliminary experiments, also consider that they have been able to dissociate allergy from immunity in experimental ringworm infections. This problem requires further elucidation.

In a recent study on human volunteers infected with certain ringworm fungi, Coombs & Kligman (30) have not been able to demonstrate the existence of a generalized acquired immunity, such as is observed in experimental animals, but in confirmation of Bloch's observations (15), have shown that reinoculation into the healed primary site fails to result in a reinfection and thus there is in this circumscribed area, an apparent immunity. This situation appears to differ only in degree from that described by DeLamater (26) in experimental ringworm.

## Immunology of the Systemic Mycoses

Even less is known about immune mechanisms in the systemic mycoses than in the superficial mycoses. A remarkable feature of these diseases is their singular lack of communicability from person to person. By and large, it would appear that natural resistance to the systemic pathogens is of a high order, but on the other hand, one might as well explain the relative rarity of systemic mycoses in humans on the basis of low virulence on the part of the pathogens.

The fungi which cause systemic disease constitute an aggregation of dissimilar fungi. For detailed consideration of these organisms the reader is referred to the standard works (3, 37, 38, 39). A relatively detailed account of the immunology of the more important diseases will be presented under separate headings for each disease.

For purposes of discussion, the systemic fungi may be placed in two groups:

(*a*) endogenous, including *Actinomyces bovis*, *Candida albicans*, and possibly (?) *Cryptococcus hominis* (*neoformans*). These fungi may occur normally on or within the human body.

(*b*) exogenous, divided into (i) wound pathogens, including *Sporotrichum schenckii* and *Hormodendrum pedrosoi*. Diseases produced by these fungi frequently follow lacerations or wounds; (ii) those fungi producing primary pulmonary diseases, which may or may not become disseminated to other organs. The primary or initial infection is most often transitory and benign. The group includes *Coccidioides immitis* and *Histoplasma capsulatum*.

*The id phenomenon*.—The id is a sterile skin lesion of very variable nature which results from the hematogenous dissemination of fungus antigens. It is an allergic manifestation occurring in highly sensitized individuals with a demonstrable fungus infection. Ids are not infrequently observed in the superficial ringworm infections, particularly those which have a marked inflam-

matory character (3, 40 to 44). Injections of trichophytin into humans with inflammatory ringworm may precipitate the id reaction (45). Ids or id-like reactions may also be experimentally produced in sensitized animals (46, 47). Allergic skin reactions may also develop spontaneously in certain of the deep mycoses. The best example is the appearance of erythema nodosum in about 5.0 per cent of individuals with coccidioidomycosis.

*Torulosis and Cryptococcus neoformans.*—Torulosis is one of the few systemic mycoses in which it has not been possible to demonstrate cutaneous sensitivity to the fungus or its products in humans or animals (48). Agglutinins have been reported in the serum of a patient only once (49), and it is generally thought that circulating antibodies are not produced during the course of human infection (50 to 53). This is also true of infected dogs, guinea pigs, rabbits, and mice (54).

The organism appears to have a relatively low antigenic capacity. The presence of agglutinins in the sera of experimental animals following hyperimmunizing injections of large quantities of living or dead cells has been reported by some and denied by others. Benham (55) was the first to enjoy success consistently. She observed in rabbits an agglutinin titer of 1:160 following a series of 10 to 20 weekly injections of killed cells. By means of serologic reactions Benham was able to demonstrate that the organisms classified in her Group III, of which *C. neoformans* is the prototype, are serologically distinct from other saprophytic species of *Cryptococcus*. These latter, interestingly enough, have high antigenic capacities.

In Benham's Group III are included nonpathogenic organisms which may be isolated from normal skin and feces and which are morphologically and immunologically similar to *C. neoformans*. Members of Benham's Group II also appear remotely related to *C. neoformans*. It is tempting to assume that such organisms may spontaneously acquire virulence in some unknown fashion. This would explain the source of the organism. Ravits (56) encountered no difficulty in isolating Group III cryptococci frequently from normal and diseased skin.

Kligman (57) was unable to demonstrate agglutinins in animals following hyperimmunization with his three strains of *C. neoformans;* however, undiluted serum was not tested. Cox & Tolhurst (48) with several strains were able to produce titers of only 1:4, but one strain yielded a titer of 1:28. Strain differences may account for the dissimilar findings of various investigators. Salvin (personal communication) reports little difficulty in obtaining high titers with his strains in rabbits, provided adequate doses of antigen are used.

In a recent study Evans (58) immunized rabbits with nine strains of *C. neoformans*, employing large injections of whole cells thrice weekly for a total of 12 weeks. Against three strains the titers reached 1:320. Four other strains incited titers of 1:10 to 1:40. Agglutinins could not be demonstrated against the two remaining strains. Employing the technique of reciprocal absorption of agglutinins, Evans arbitrarily divided his strains into three immunologic types, designated A, B, and C. It is obvious, therefore, that the

production of agglutinins depends upon (*a*) the strain, and (*b*) the repeated injection of large quantities of antigen. The problem has been further resolved by Neill and his colleagues (59), who point out that agglutinins can be consistently observed in animals following 13 daily injections of dense suspensions of weakly encapsulated strains. Titers of 1:160 have been attained in this fashion. Strains characterized by large capsules are practically nonantigenic.

Kligman (57) identified the capsular material as a polysaccharide and isolated it in a crude state. Neither precipitins nor skin sensitivity could be induced in animals by injections of this polysaccharide. This report gains in interest with the recent finding that the capsule of *C. neoformans* is the mucopolysaccharide, hyaluronic acid. According to Drouhet & Segretain (60), the capsule can be dissolved by treating the cells with the enzyme hyaluronidase. Since hyaluronic acid is a normal constituent of the body its lack of antigenicity is to be anticipated.

Mager & Aschner (61, 62) have reported the production, under proper cultural conditions, of an extracellular polysaccharide which stains intensely blue with iodine. This has been shown to be a carbohydrate of the amylose type. Among the polysaccharides produced by *C. neoformans* is an extracellular substance (Fraction B) which does not give a blue color with iodine and which has interesting immunologic properties (63). Neill *et al.* (64) were able to show that antiserum prepared against an unrelated strain of *Oospora* gave a precipitin reaction with fraction B from *C. neoformans* in the extraordinary dilution of 1:2,000,000 of the antigen.

The same *Oospora* antiserum could be used to demonstrate a modified Quellung reaction, employing whole cells of *C. neoformans*. The capsule of this species is ordinarily not visible when the cells are suspended in water. The addition of *Oospora* antiserum to such a suspension renders the outline of the capsule clearly visible. This change is apparently not due to a swelling of the capsule but rather to a chemical alteration which modifies the refractive index in such a manner as to make the capsular outline stand out sharply. Only one of the antisera prepared by intense immunization of rabbits with *C. neoformans* gave this modified Quellung reaction and this only with the undiluted serum. Neill and his associates (64) failed to produce agglutinins and precipitins with several strains of *C. neoformans* except in very low titers (usually undiluted serum). The antiserum prepared against the strain of *Oospora*, however, showed a marked degree of cross agglutination with *C. neoformans* in relatively high titers. The Quellung reaction may not occur with sera from human subjects since agglutinins are absent.

Several investigators were unable to protect mice against fatal torulosis by a previous course of immunization (57, 65). There is thus no evidence of acquired immunity in this disease. The possibility of immunization to *C. neoformans* with an antigenic strain of *Oospora* similar to the one used by Neill is an engaging one.

*Actinomycosis and Actinomyces bovis.*—The term actinomycosis refers to the disease produced by the anaerobic organism, *Actinomyces bovis*. Strictly

speaking, actinomycotic infection caused by aerobic actinomycetes belonging to the genus *Nocardia* should properly be termed nocardiosis. *A. bovis* is an endogenous organism which may be readily isolated from tonsils and from carious teeth (66, 67). Actinomycosis is nonetheless a relatively rare disease and one may assume that there is a high degree of natural resistance to infection.

There is a paucity of immunologic data on this disease and much of what is in the literature is contradictory. Unfortunately, experimental infection of animals is generally not possible (68).[4] Occasionally a fatal progressive type of actinomycosis can be produced in guinea pigs by repeated inoculation with live organisms. It has been suggested that the increased likelihood of infection following repeated inoculations of this sort may depend upon the development of hypersensitivity (69). Proof of this is lacking. There is no substantial evidence that allergy is a prerequisite to infection in this or any other disease. Allergy is more often thought of as an instrumental component of immunity rather than as a condition for the development of infection.

Colebrook (70) was one of the first to demonstrate agglutinins in the sera of human cases of actinomycosis; however, because of the difficulty of preparing uniform and stable suspensions, and the presence of cross reactions with other fungi, the agglutination test has not been shown to have any real value (37). Neuber (71), on the other hand, places such confidence in the specificity of the complement-fixation test with a polyvalent antigen, that he considers a positive test diagnostic of visceral actinomycosis even if the organism cannot be isolated. This is an enthusiastic view. It is dangerous to place such reliance on an immunologic test. These studies require confirmation.

The value of cutaneous tests with antigens prepared from *A. bovis* is likewise in doubt. Conant (37) considers it established that patients with actinomycosis exhibit positive skin reactions to vaccines or culture filtrates. Subcutaneous injections of the antigen may result in focal reactions and generalized symptoms. On the other hand, Mathieson *et al.* (69) reported on five patients with actinomycosis, none of whom showed significant skin reactions to intracutaneous tests with vaccine; on the other hand "positive" reactions were observed in normal individuals. At present one can hardly assume that skin reactions are specific or even helpful in diagnosis. Neuber (71) found that individuals with actinomycosis frequently failed to react to the vaccine injected intracutaneously. The current value of any immunologic procedure in the study of actinomycosis is compromised by these contradictory findings.

Neuber recommends a course of vaccine injection as a therapeutic measure in individuals with actinomycosis, and contends that death is practically inevitable in those individuals who remain anergic. On the other hand,

[4] A personal communication from Dr. Esther Mayer of the University of Illinois, College of Medicine, indicates that infection of mice may be regularly accomplished when the organism is first suspended in gastric mucin. We have confirmed this. The availability of this technique reopens immunologic investigations with this organism.

sensitized individuals are said to benefit greatly from injections of polyvalent vaccines. This work also requires confirmation. Active immunization with the causal organism during the course of infection has not found acceptance as a therapeutic measure in chronic infectious diseases in general.

Erikson (72), contrary to majority opinion, considers that there are two distinct species of *Actinomyces*. She found that the human species (*A. israeli*) was antigenic, while the bovine organism (*A. bovis*) was not. This requires further study.

*Nocardiosis and Nocardia asteroides.*—The pulmonary disease caused by *N. asteroides* may rather closely resemble tuberculosis. About half the cases of an unrelated mycosis, mycetoma (Madura foot), are caused by several different species of *Nocardia*. Immunologic data on mycetoma are lacking. Only the pulmonary disease caused by *N. asteroides* will be discussed here.

*N. asteroides* has been isolated from the soil (73). It is an exogenous organism. Again, as with *A. bovis*, the failure to infect experimental animals consistently is a limiting factor in immunologic studies with this organism. Evidently strains differ in virulence (38). Although Drake & Henrici (74) were unable to produce chronic progressive disease with their isolates, others have found their strains highly virulent for rabbits and guinea pigs. Mathieson *et al.* (69), working with a virulent strain, determined that cutaneous sensitivity to a vaccine can be established in guinea pigs and rabbits by immunization and also by infection. Long-continued immunization is said to lead to desensitization. This fungus has many morphologic and even immunologic similarities to the tubercle bacillus. By means of the complement-fixation test Nelson & Henrici [quoted by Skinner *et al.* (38)] demonstrated cross reactivity between these two species. Mathieson's sensitized guinea pigs failed to show a cutaneous cross reaction to tuberculin. Drake & Henrici confirmed this and further reported that tuberculous animals do not react to a *Nocardia*, antigen.

Of particular interest in this regard is a recent report by Freund & Lipton (75) who were able to sensitize guinea pigs strongly with *N. asteroides* prepared in a water and oil emulsion. These animals exhibited clear-cut cross reactions to tuberculin, although the cutaneous responses were minimal. There is, thus, evidence of a moderate degree of cross reactivity between these two organisms which is probably not of such an order as to have clinical significance.

An unexpected application of *N. asteroides* in immunologic studies has recently emerged from the work of Freund & Lipton (75). It has been well established that the presence of tubercle bacilli in a water and oil emulsion (adjuvant mixture) greatly enhances the development of sensitization to drugs, such as picryl chloride (76). Iso-allergic encephalomyelitis may also be produced in experimental animals by incorporating tubercle bacilli in an appropriate adjuvant mixture (77, 78). *N. asteroides* may be substituted for the tubercle bacillus without loss of potency in procedures involving immunization with adjuvants, as described above. The substitution of *N. asteroides* avoids the incidental sensitization to tuberculin which invariably results when tubercle bacilli are incorporated in the water and oil emulsions.

Drake & Henrici's (74) work assumes new significance in the light of these findings. These investigators demonstrated cutaneous sensitivity to protein and polysaccharide antigens of *N. asteroides* following injections of rabbits intratesticularly with a suspension of the live organisms in oil. Although they attributed their success with this method to the development of a foreign body granulomatous reaction brought about by the oil, it is possible that they anticipated with this technique the later work of Freund with adjuvant mixtures. The sensitized guinea pigs of Drake & Henrici also gave positive cutaneous reactions to defatted powdered organisms. The skin test principle was not present in the filtrates. Nonspecific reactions occurred when the organisms were not defatted with lipoid solvents. The skin testing antigen was found to be heat labile.

Glover and his associates (79) recently demonstrated skin sensitivity of the delayed tuberculin type in a patient with *N. asteroides* infection. The specificity of the reaction is not known since the disease is rare.

*Moniliasis and Candida albicans.*—The causative organism of this infection occurs in the mouth and gastro-intestinal tract of about 15 per cent to 50 per cent of normal individuals (3, 80). Although *C. albicans* is really the only common pathogenic species in the genus *Candida*, the opportunistic nature of fungus pathogens is well illustrated by the rare endocarditis caused by *Candida parakrusei* in drug addicts (81, 82). The latter species is ordinarily nonpathogenic. Disease apparently results from the depression of host resistance observed in addicts who are often in a poor nutritional state.

The commercial material for intracutaneous testing for sensitivity to *C. albicans* is known as oidiomycin. The reaction is of the delayed tuberculin type, but it has little diagnostic value. Lewis *et al.* (83) report that 43 per cent of individuals with cutaneous moniliasis fail to react to oidiomycin, and observe that 46 per cent of positive reactions were elicited in 192 individuals who showed no evidence of disease. Lewis & Hopper (3) recommend the abandonment of the oidiomycin skin test.

Conant and his associates (37) agree that the test has no diagnostic value, at least in cutaneous moniliasis. Positive patch tests may be elicited in sensitive individuals (84). The large percentage of positive reactions in normal individuals who have never had moniliasis undoubtedly results from the fact that *C. albicans* or antigenically related species are wide-spread in the normal population. However, there is no evident relationship between skin sensitivity and the endogenous presence of *Candida* in the gastro-intestinal and respiratory tracts. The experimental study of sensitivity is limited by the fact that moniliasis similar to that observed in humans cannot be produced in animals. Owen, Anderson & Henrici (85) were able to establish cutaneous sensitivity to *C. albicans* in rabbits by intravenous injections of living cells. The cutaneous reaction, however, could be elicited only by the intradermal injection of living cells. Cross-sensitivity to other species of *Candida* and to the common yeast, *Saccharomyces cereviseae*, was demonstrated.

Weiss & Landron (86) also found that positive skin reactions did not occur when heated cell suspensions were used as antigens in rabbits immunized by intravenous injections. Skinner (80), however, states that repeated injec-

tions of killed cells nearly always sensitize animals. A polysaccharide said to be isolated from the capsule of the organism (the capsule has not been clearly demonstrated to our knowledge) gives positive cutaneous reactions in individuals sensitive to oidiomycin (87, 88, 89). A number of polysaccharides have evidently been isolated (90, 91).

Following the repeated injection of killed cells of *C. albicans* into rabbits, the intravenous injection of polysaccharide results in anaphylactic shock (92, 93). The anaphylactic state cannot be established by immunization with the polysaccharide alone. Guinea pigs may be rendered passively anaphylactic by injections of rabbit sera. With this latter technique anaphylaxis to other species of *Candida* and to the unrelated *S. cereviseae* may be produced.

Mott & Kesten (94) found with *C. albicans* that the intravenous injection of purified polysaccharide into animals whose anterior eye chambers had previously been inoculated with whole cells, resulted in a pronounced inflammatory reaction in the inoculated eye. There appears to be little doubt that the antigen is closely associated with the polysaccharide fraction.

Kurotchkin & Lin (95) believe they were able to cause bronchomoniliasis in rabbits by the repeated injections of *Candida tropicalis* into the trachea. This experiment evidently reflects the view that sensitivity predisposes to or is a requisite of infection. In view of the fact that the rabbits were killed two weeks after the last injection and smears were negative for fungi, it might appear that the nodules observed by these investigators in the rabbit lungs may have been allergic tissue manifestations rather than evidence of actual parasitism.

Moniliids have been described in humans (96, 97). These are allergic skin manifestations to *C. albicans* and are similar to the better known trichophytids. The injection of oidiomycin into a sensitized person may reproduce these allergic lesions. The criteria for making a diagnosis of moniliid should be strict. A positive oidiomycin test in a patient with obscure skin lesions is not sufficient evidence. The organism must be isolated from an active primary focus.

Agglutinins may be readily demonstrated in the sera of humans and experimental animals. Because of the lack of clear-cut end points in the agglutination reactions, Martin (98) prefers the complement-fixation test. The agglutination test has been said to be compromised by the tendency of the organisms to clump spontaneously. Norris & Rawson (99) have overcome this objection by using cell suspensions from 24-hr. cultures. They recommend the volumetric titration of the antigen with anti-*Candida* rabbit serum. Repetition of their work indicates that the agglutination test is satisfactory when used according to their specifications.

The occurrence of agglutinins in the sera of normal individuals has received considerable attention. Todd (100) found that 22.5 per cent of normal individuals had titers of 1:10 or higher. (The lowest serum dilution used was actually 1:20.) Of 1,150 individuals only eight had titers of 1:320 and one a titer of 1:640, the highest found. Drake (101), employing a slide agglutina-

tion test, found detectable titers in 89 per cent of normal individuals. Fifty-three per cent of the same group had agglutinins for the common yeast, *S. cereviseae*. He reported that 63 per cent of normal rabbits have anti-*Candida* agglutinins. Drake's lowest serum dilution was 1:2, which undoubtedly accounts for his high figures.

Norris & Rawson (102), using their improved agglutination technique, report that 64 per cent of normal individuals have titers of 1:5 or greater. In women, the highest titer was 1:320 and in men, 1:180. In a small number of children no titers higher than 1:10 were found. They confirmed the presence of cross-agglutination to *S. cereviseae*. The findings of Fuentes & Guarton (103), who were able to demonstrate agglutinins in a titer of 1:20 or higher in only two of 1,002 unselected cases, are not consistent with the observations of others. Agglutinins have been reported in patients with bronchopulmonary disease from whom *C. albicans* was isolated from the sputum (104 to 107).

The question naturally arises as to the diagnostic value of the agglutination tests. Conant *et al.* (37) state that they have encountered severe infections (they evidently refer to skin and mucous membrane lesions) in which the agglutinin reaction was negative. Some of their other cases had agglutination titers of 1:2400. They dismissed the agglutination test as valueless in the diagnosis of moniliasis.

In a limited number of cases the authors have observed inconstant agglutination titers in cases of extensive cutaneous moniliasis. It is possible that the agglutination test has no place in the cutaneous form of this disease. The value of the test in pulmonary moniliasis is still to be determined. Although it is true that agglutinins are found in the sera of normal individuals, the titers are low. In children, for instance, the titers do not exceed 1:10, and in adults titers higher than 1:80 are decidedly uncommon. If we regard these figures as maximal for the normal range, titers considerably higher than these may have some diagnostic significance. The authors have observed two cases of meningo-encephalitis in children due to *C. albicans*. In one the agglutination titer was 1:320 and in the other it was 1:640. In an adult with extensive pulmonary disease the titer was repeatedly 1:32,000. The impression persists that such extremely high titers are meaningful when found. The problem requires further elucidation.

Pulmonary moniliasis is a difficult disease to diagnose. The isolation of the organism has little diagnostic significance because of its endogenous character. Likewise, *C. albicans* commonly occurs in association with other chronic pulmonary diseases, such as carcinoma, lung abscess, bronchiectasis, etc. A valid immunologic test for differentiating primary from secondary moniliasis would be most welcome.

By means of agglutination tests the existence of common antigens among species of *Candida*, *Hansenula*, *Endomyces* and *Saccharomyces* have been demonstrated (108 to 111). Curiously enough, anti-*C. albicans* rabbit serum fixes complement with *Histoplasma capsulatum* and *Blastomyces dermatitidis* antigens (112). Anti-*Histoplasma* and anti-*Blastomyces* sera do not, however,

fix complement with *Candida* as the antigen. There is at least one common antigen amongst these species. By means of precipitin absorption studies Martin (98) was able to show that *C. albicans* and *C. parakrusei* are antigenically separable, although they possess a common antigen also.

*C. albicans* and *C. stellatoidea* are antigenically similar. This is of real interest since it supports the view that *C. stellatoidea* is simply a variant of *C. albicans.*

Finally, a case report by Hiatt & Martin is of immunological interest (113). They describe a patient with pulmonary moniliasis who failed to react to intradermal test with an autogenous vaccine, and who had no agglutinins in her serum. However, the injection of anti-*Candida* rabbit serum resulted in a severe cutaneous reaction (the Foshay test). Following the injection of an anti-*Candida* serum in increasing doses, there was a reversal of the skin reaction to positive, agglutinins appeared in the serum, and there was a complete remission of symptoms. Smith (114) mentions another case in which the immunologic findings were similar and in which serum therapy produced the same clinical result. An analogous immunological situation has also been encountered in blastomycosis and will be discussed below.

It should not be assumed from these cases that immunization with an immune serum is an accepted form of therapy.

*Sporotrichosis and Sporotrichum schenckii.*—The causative organism is a classical example of a wound pathogen. In addition to the numerous cases reported from the South African gold mines (115), Foerster (116) has reported a remarkable series of eighteen cases in fourteen of which infection followed wounds by barbary thorns. Since the organism is widespread in nature, natural resistance to infection must be considered high. Human infection would appear to be an incidental phase in the life cycle of the organism.

Widal and his associates (117) first demonstrated agglutinins and complement-fixing bodies in the sera of infected humans, a finding which has been repeatedly confirmed (118, 119). However, cross-agglutination with sera from cases of thrush and actinomycosis has been reported (117). This latter observation has not been confirmed by Fineman (120) in his studies on patients with thrush. The serum of normal individuals may cause agglutination in low titers (121), as confirmed by the current authors. Undiluted rabbit serum will agglutinate a suspension of sporotrichum cells (64). The same phenomenon has been observed with undiluted guinea pig serum.

The agglutination studies of earlier investigators employed cell suspensions of the mycelial phase. Superior suspensions for the agglutination test may be prepared from the yeast-like phase grown on cystein blood agar (122). The specificity of the agglutination reaction is, however, undetermined. Agglutinins and precipitins may readily be developed in guinea pigs and rabbits by routine immunization procedures (123). It would appear that the test gains value when the antigen is first volumetrically titrated to determine the optimal concentration. Anti-sporotrichum serum does not fix complement in the presence of *Histoplasma* and *Blastomyces* antigens (124).

Cutaneous sensitization to a vaccine or an extract of the fungus occurs

regularly in individuals with sporotrichosis (121). The reaction is of the delayed tuberculin type. Cutaneous sensitivity is such a constant feature that DeBeurmann (118) considers that sporotrichosis may be ruled out as a diagnosis when the skin test is negative. Cutaneous reactions are often severe. Focal reactions with malaise, lymphadenopathy (119), etc., have been reported. The existence of cutaneous cross-reactions with other fungus antigens has not received thorough study. The evidence to date, however, suggests that the reaction is fairly specific. Cutaneous sensitivity has been observed to develop within five days after the experimental inoculation of a human volunteer (121).

Sporotrichids, similar in nature to trichophytids, have been observed (118). They are evidently uncommon.

DeBeurmann & Gougerot (125) were unable to demonstrate the existence of an acquired immunity in humans reinoculated with the organism.

Lurie (123) collected sixteen isolates of *Sporotrichum* from all over the world, including several supposedly different species, and found them to be antigenically similar by agglutination and agglutination absorption tests. This finding supports the contention that there is only one valid species of *S. schenckii*.

Neill *et al.* (64) have described a Quellung reaction, employing yeast cells obtained from infected animals. When such cells are mixed with specific anti-serum a distinct swelling of the capsule occurs. Unfortunately, the reaction is not demonstrable using the yeast phase organisms grown on cystine blood agar. For proper performance of the test one must collect organisms from the peritoneum of infected mice, and it must be appreciated that many of the cells will have a capsule even before admixture with the antiserum. It is unlikely that the Quellung reaction will have any practical diagnostic significance. Fortunately however, the diagnosis of sporotrichosis is easily made.

*Blastomycosis and Blastomyces dermatitidis.*—As a by-product of the intense study which histoplasmosis is currently receiving in this country, there has been a considerable accumulation of immunologic data on blastomycosis. This disease occurs in a cutaneous form from which recovery is frequent, and a visceral form which has a far more serious prognosis.

Stober (126) first noted that infected individuals become allergic to the fungus. This has been repeatedly confirmed (127, 128). The major observations of this disease have been made by Martin & Smith at Duke University (129, 130, 131). Skin sensitivity may be tested either by the intradermal injections of the killed yeast-phase vaccine (principally employed by Martin & Smith) or the use of blastomycin. This latter is a culture filtrate antigen prepared in a manner similar to that of histoplasmin and coccidiodin. The culture filtrate has not given as consistent results as the vaccine in the hands of most workers.

The time required for the skin test to become positive following infection is unknown. The skin test may become negative in a previously sensitized individual in the terminal phases of the disease (131). This is part of a general

depression of cutaneous sensitivity and has been recognized to occur in tuberculosis, histoplasmosis and coccidioidomycosis (132, 133) and other diseases. Rarely cutaneous sensitivity may fail to become established during the entire course of the disease (130). Martin (1) was so impressed with the diagnostic value of positive cutaneous tests that he wrote in 1941: "Although a negative reaction to *Blastomyces dermatitidis* does not exclude the possibility of blastomycosis, a positive reaction is diagnostic of the disease, and we have not as yet obtained a positive reaction for patients with other disease."

Cross-sensitivity between histoplasmin and blastomycin is a well established phenomenon in animals (134, 135). Animals with blastomycosis show practically a 100 per cent cross reaction to histoplasmin. Conversely, only about half of the animals infected with *Histoplasma capsulatum* react to blastomycin. Blastomycin would appear to be the more specific antigen from the standpoint of the skin test. The cross sensitivity between *Coccidioides immitis* and *B. dermatitidis* is of a low order.

In human beings a positive blastomycin test may result from infection with a heterologous fungus. Thus, strong coccioidin reactors tend to show a positive blastomycin test (136). One of the patients in Bunnell & Furcolow's (133) series of ten proven cases of systemic histoplasmosis also reacted to blastomycin. Upon appropriate dilution of the antigen the skin reactivity to blastomycin was not elicited, although the histoplasmin test continued to be positive in an equivalent dilution. Because of this cross reactivity a single skin test may be misleading. A "titration" of the antigens by dilution, as recommended by Howell (135), will reveal the homologous antigen because the reactivity to the homologous antigen persists at higher dilutions than with heterologous antigens. Skin tests with all the cross reacting antigens should be made at the same time. The homologous antigens will generally show the most intense skin reaction, but if there is any doubt a titration of the antigens should be performed. The value of this procedure has been clearly demonstrated in animals.

There is general agreement that the cross reactivity is greatest between histoplasmin and blastomycin. That exhibited by either of these antigens with coccidioidin is of a lesser order.

Howell (135) has suggested that an antigen should be employed at its critical titer in order to avoid cross reactions. He defines the critical titer as that minimal amount of homologous antigen which would detect sensitivity in approximately 80 to 90 per cent of a group of infected animals. It is assumed that the heterologous antigen would probably fail to react at this critical titer. Unfortunately, the problem of cross reactivity probably cannot be resolved in this fashion. At the present time neither the antigens nor the sensitivity of the animals can be sufficiently standardized. It is well known, in humans as well as in animals, that the sensitivity may vary with the stage and extent of infection. Moreover, the currently available antigens are crude and vary from lot to lot. Cross & Howell (137) prepared a polysaccharide from *Histoplasma capsulatum* which still gave cross reactions in

*Blastomyces* infected animals. It is possible, of course, that further chemical purification of the antigens may partially or wholly eliminate the cross reactions.

The cutaneous reaction to blastomycin is of the delayed tuberculin type. Positive skin reactions in patients with blastomycosis may be elicited with both polysaccharide and protein fractions isolated from the organism (138). Following intravenous injection of the polysaccharide, the patient may become temporarily desensitized. Occasionally the skin reaction is of the immediate urticarial type (130). Such reactions have also been observed with trichophytin and are frequently associated with the presence of the circulating antibodies which can be passively transferred by the Prausnitz-Küstner method (8 to 12).

Complement-fixing antibodies are frequently detectable in the sera of patients with extensive blastomycosis (131, 139). The titer of the complement-fixing antibodies corresponds roughly with the degree of internal involvement by the disease (1). Extensive skin lesions, on the other hand, may fail to produce complement-fixing antibodies. The complement-fixing test thus appears to have prognostic value because of its greater likelihood of being positive in generalized cases. The complement-fixation test described by Martin (139) employs a suspension of the fungus cells in the yeast phase as an antigen. The test is relatively insensitive and the antigen tends to deteriorate fairly rapidly. The complement-fixation test for histoplasmosis of Campbell *et al.* (140) using a ground yeast phase antigen, is probably superior. It has been successfully used in animal blastomycosis. The complement-fixation tests described for histoplasmosis by other investigators are equally satisfactory when *Blastomyces* is substituted as the antigen (141 to 144). The optimal dilution of antigen should first be determined. This has been defined by Saslaw & Campbell (141) as the greatest concentration contained in 0.2 ml. beyond which further increase fails to enhance the serum reaction. This dilution must, of course, be neither hemolytic nor anticomplementary.

*B. dermatitidis* has poor immunogenic capacities as revealed by complement-fixation tests in animals and in humans. The titers which can be obtained in experimental animals are low. In humans a positive complement-fixation test, even with undiluted serum, is presumed to have some diagnostic value. According to Martin (1), the Duke investigators have not observed a positive complement-fixation reaction in a patient without blastomycosis. Bunnell & Furcolow (133), however, have reported a number of proved cases of human histoplasmosis in which cross complement-fixation reactions to *Blastomyces* were observed. This cross reactivity parallels that which has been described for cutaneous tests. It is significant that the homologous antigen can be detected by titration of the antisera in much the same fashion as the homologous antigen is detected in cases of cutaneous cross reactivity by titration of the antigen (133). This means that quantitative complement-fixation tests should be made. Thus, for diagnostic purposes both antigens should be used in the complement-fixation tests. Titers ob-

tained with anti-*Histoplasma* sera, using *Blastomyces* as the antigen, are generally higher than those obtained when a *Blastomyces* antigen is tested in the presence of its own antiserum (112, 141). Nonetheless, anti-*Blastomyces* serum still shows a higher titer with its specific antigen than when *Histoplasma* is the antigen. The titer of anti-*Histoplasma* serum in the presence of its specific antigen is greater than when *Blastomyces* is the antigen. Anti-*Histoplasma* serum does not fix complement in the presence of *B. brasiliense.* This is curious since this latter organism appears to be closely related to *B. dermatitidis.* The immunologic relationships between these two organisms require further study.

The collodion agglutination technique of Cavelti (145) has been adapted to the detection of anti-*Blastomyces* antibodies (146, 147). The collodion particles are sensitized with blastomycin. While the results are clear-cut in animals, studies on human sera have not yet been reported. The test has much to recommend it. The cross sensitivity between *Histoplasma* and *Blastomyces* is, however, not eliminated.

On the basis of his experience with human blastomycosis, Smith (131) has attempted to classify his cases into four immunologic types. This represents an attempt to exploit immunologic studies at a clinical level for use in prognosis and therapeutic management. His series is small for such extensive conclusions to be drawn, and extension of these studies is most desirable. The individuals in Smith's Group I have the best prognoses. These cases either had cutaneous blastomycosis or a recent pulmonary involvement. The skin test was positive and the complement-fixation test was negative. In his Group II he includes individuals who are skin-test positive and who also have a positive complement-fixation test. The disease is more extensive than those in Group I and the prognosis is poorer. In Group III are placed the individuals with negative skin tests and positive complement-fixation reactions. These patients are almost always in the terminal stages of the disease. They exhibit a negative anergy. In the last group (IV) are included the individuals who are skin-test negative and whose complement-fixation test is also negative. From the standpoint of classification this is a very unsatisfactory group, since it includes individuals whose clinical conditions are diverse, some being in the terminal phase of the disease, others very early infections, and still others unusual cases that are clinically nonreactive. Blastomycosis, therefore, cannot be excluded on the basis of a negative skin test, nor on the basis of a negative complement-fixation test.

Martin & Jones (148) report a case, which would fall into Smith's Group IV, which is basically similar to the case of moniliasis described by Hiatt & Martin (113). The intracutaneous injection of anti-*Blastomyces* rabbit serum caused an immediate reaction. Following a series of injections of rabbit antiserum the patient's immunologic picture was reversed. He became skin-test positive, developed a positive complement-fixation test, and subsequently recovered. Such cases are exceedingly rare and are difficult to explain. One should not assume because of the recoveries reported in such cases that injections of antiserum and of autogenous vaccines are dependable forms of treatment.

Martin & Smith (130) caution against the therapeutic use of iodides in patients with cutaneous sensitivities, since this may be followed, according to them, by a rapid extension of the disease and even death. They urge desensitization of such patients with a *Blastomyces* vaccine following which iodides may be given. The paucity of such cases compromises the validity of their thesis. Nor are the authors entirely consistent in representing their viewpoint on the adverse effect brought about by iodides in allergic patients. For instance, they had previously reported a case of blastomycosis in a young Negro male (Case 3) whose skin test was only weakly positive. Despite the low grade sensitivity of this patient, death occurred within a month following treatment with iodides. Smith (131) further reviews a case history in which there was neither cutaneous sensitivity nor circulating antibodies, yet this individual died following a course of iodides. Although this patient did well for a while, generalization of the lesions suddenly occurred. This obviously could not have been due to any destructive allergic effect potentiated by the iodides since the patient was not sensitive. But therapeutic failure in this case in a patient without circulating antibodies is hypothetically attributed to the administration of iodides. Accordingly, Smith empirically recommends vaccine therapy for such individuals until the complement-fixation test becomes positive, after which iodides may be administered. It would appear that the Duke workers tend to attribute an adverse course to the harmful effect of the iodides, in some instances representing that this effect is mediated through allergy, and in other instances through the lack of circulating antibodies. One is compelled to remark that iodides are not a specific form of therapy for blastomycosis and that some deaths will occur irrespective of the immunologic status of the patient. The issue cannot yet be settled. Smith's (131) work constitutes a major contribution, but his recommendations are derived mainly from empirical findings in a small group of cases.

*Histoplasmosis and Histoplasma capsulatum.*—The immunologic approach to the study of this disease, particularly by means of skin testing, has proved fruitful. Recent studies have revealed that there exists in the general region of the Mississippi Valley basin an area in which there are many individuals with pulmonary calcification who are, nonetheless, nonreactors to tuberculin. Extensive skin testing with histoplasmin in this area has shown that: (*a*) there are more reactors to histoplasmin than to tuberculin, and (*b*) there is a higher correlation between histoplasmin sensitivity and pulmonary calcification than exists between tuberculin sensitivity and the presence of calcification (149 to 153). Pulmonary calcification is three to five times more frequent in those who react only to histoplasmin than in those who are pure tuberculin reactors. The data excludes the possibility of cross reactions between histoplasmin and tuberculin. A strong case has been made for the existence of a benign subclinical form of histoplasmosis which renders the majority of individuals in the endemic area sensitive to histoplasmin. Many individuals originally nonreactive to histoplasmin have been observed to develop pulmonary calcification with simultaneous conversion of the histoplasmin reaction to positive (154, 155). Similar cases have now

been observed which had positive cultures for *H. capsulatum* during the period of active disease. It should be pointed out that the combined groups of tuberculin- and histoplasmin-positive individuals do not account for all the pulmonary calcifications observed.

Histoplasmin sensitivity becomes established at an earlier age than does tuberculin sensitivity (156). Sontag & Allen (157) have observed histoplasmin sensitivity and pulmonary calcification to develop in children at 10½ months of age. In a group of 64 such cases High *et al.* (158) showed that none reacted to tuberculin alone, while 93½ per cent reacted to histoplasmin. The incidence of histoplasmin reactors in the population falls off as one proceeds away from the endemic area (149). In North Dakota and South Dakota, for instance, the incidence is only 1 per cent. Among 365 Aleutes of the Pribilof Islands, only one native reacted to histoplasmin (159).

Despite the massive data suggesting the existence of a benign form of pulmonary histoplasmosis as a cause of pulmonary calcification, final proof is lacking. The organism has only rarely been isolated from such pulmonary cases. Pulmonary calcification occurs also in individuals who are non-reactors to both histoplasmin and tuberculin (157).

Systemic histoplasmosis, on the other hand, is a rare disease. Van Pernis, Benson & Holinger (160) first showed histoplasmin sensitivity in a patient with the systemic form of the disease.

Histoplasmin is a culture filtrate antigen similar to coccidioidin. The method of preparation has been described by Emmons (134). Salvin & Hottle (161) have found that the substitution of starch or glycogen in the medium for glucose greatly enhances the formation of histoplasmin. For unknown reasons some lots of histoplasmin are not potent and fail to give positive reactions in sensitive individuals. A means of standardization is greatly to be desired. It has been found that there is a close parallel between the capacity of a given filtrate to evoke positive reactions and to act as an antigen in the complement-fixation tests (161). Thus, the filtrates which fail to give cutaneous reactions fail to fix-complement. Salvin & Hottle suggest, therefore, that the complement-fixation test with known anti-*Histoplasma* serum is a useful method of assaying the principle in culture filtrates. In such filtrates the formation of histoplasmin reaches a maximum in about two months. The histoplasmin reaction is of the delayed type.

Vollmer (162) prepared a histoplasmin patch test material similar to that used for the Vollmer tuberculin patch test. Unfortunately, the patch test does not elicit positive reactions in all individuals who give strong reactions to the intradermal injection of histoplasmin. The intradermal reaction to histoplasmin is not inhibited by antihistaminic drugs (163). Furcolow & Ruhe (164) found that histoplasmin sensitivity among cattle in Kansas paralleled that in humans. On the other hand, in 837 dogs which were skin tested with histoplasmin, there were only five positive reactions (165). Three of these five animals had demonstrable histoplasmosis on autopsy. In this case the histoplasmin test could be construed as having greater diagnostic significance in dogs than in humans, but this is probably more apparent than real.

Cutaneous reactions in infected animals may also be elicited by a par-

tially purified polysaccharide (137). Scheff found that infected animals reacted positively to a protein and polysaccharide fraction obtained from *Histoplasma capsulatum* (166). The reaction was positive only in infected animals and not in immunized ones.

The problem of cross-reactivity between histoplasmin and other fungus antigens has already been described in the discussion on blastomycosis. Cutaneous cross-reactivity between histoplasmin, blastomycin and haplosporangin in animals is of a high order (134). The cross-reactivity is less with coccidioidin.

The accumulated data indicate that as a skin testing antigen histoplasmin is relatively nonspecific. For instance, practically all experimental animals infected with *Haplosporangium parvum* and *Blastomyces dermatitidis* react to histoplasmin. On the other hand, histoplasma-infected animals show no cross-reactions to haplosporangin; and only about 50 per cent a cross-reactivity to blastomycin (134). Much the same thing has been observed in humans. Thus, only 20 per cent of the individuals with a 1+ cutaneous reaction to histoplasmin will react positively to coccidioidin; conversely, of those with a 1+ coccidioidin reaction, 73 per cent react to histoplasmin. Strong coccidioidin reactors show 100 per cent cutaneous sensitivity to histoplasmin (136). The results of skin testing in patients with the systemic form of histoplasmosis are of great importance, since in these cases the etiology of the disease is definitely established. In ten proved systemic cases Bunnell & Furcolow (133) found seven reactors to histoplasmin. Two of the non-reactors were in the terminal stage of the disease, and the third had a fever of 105° F. at the time of the test. The loss of the sensitivity under such circumstances is a recognized phenomenon in blastomycosis, coccidioidiomycosis and tuberculosis. In one of these cases there was a cross-reaction to blastomycin, but on dilution of the antigen the blastomycin test became negative while the histoplasmin reaction persisted at the equivalent dilution. There is, thus, good evidence that cutaneous sensitivity to histoplasmin is a more or less constant feature of the disease. While a positive reaction is scarcely diagnostic, repeatedly negative reactions in cases without fever and not in the terminal phases of the disease argue against the diagnosis of histoplasmosis.

The complement-fixation reaction has been extensively studied in animals and humans. A number of different tests have been described using as antigens histoplasmin, whole yeast cells, ground-up yeast cells, and the residue of ground yeast cells (140, 141, 143, 144, 167). The test described by Campbell & Saslaw (140), using ground yeast cells, has proved the most satisfactory in our hands. Campbell (personal communication) indicates that the latter antigen can probably be preserved indefinitely following lyophilization. The ground yeast phase antigen can be easily prepared by grinding the cells in a mortar with dry ice and sand. Regardless of the antigen used, the optimal dilution should first be determined, preferably with known human anti-*Histoplasma* serum. Salvin & Hottle's (167) important work in animals has elucidated some of the factors which must be recognized in attempting to use immunologic reactions in the study of this disease. These are: (*a*)

the nature of the antigen, (*b*) the stage of the disease, and (*c*) whether the animal is infected or immunized.

The complement-fixation titers are considerably higher in hyperimmunized animals than in infected ones. The complement-fixation reaction reaches a peak in about five weeks and persists at a fairly constant level for about six months. Salvin & Hottle (167) investigated the suitability of a number of antigens in the complement-fixation test. These were essentially of two types: (*a*) particulate antigens which included ground mycelium, whole yeast cells, and the residue of ground yeast cells, and (*b*) soluble antigens which included culture filtrates. In hyperimmunized animals the highest titers were obtained with the soluble antigens. On the other hand, in infected animals the particulate antigens gave the highest titers and the culture filtrates were completely inactive. Despite this, positive complement-fixation reactions have been reported in humans using histoplasmin (a soluble antigen (142) and the ground yeast phase antigen of Campbell & Saslaw (168), which is also probably a soluble antigen. Further study is required to resolve this dilemma. At any rate, the antigens elaborated by the yeast and the mycelial phases are immunologically similar.

Anti-*Candida albicans* and anti-*Coccidioides immitis* sera will fix complement in the presence of a *Histoplasma* antigen (112). Anti-*Histoplasma* serum, on the other hand, does not fix complement with *Candida albicans* or *Coccidioides immitis* as the antigen. The cross reaction between *Coccidioides* or *Histoplasma* in the complement-fixation test is of a minor order and not likely to interfere with the validity of the reaction in these diagnoses. The cross reactivity exhibited by these various antigens in complement-fixation tests parallels the cross reaction exhibited on intracutaneous testing. As an antigen capable of producing relatively specific antisera, H. *capsulatum* enjoys a greater degree of specificity than it does as an antigen for use in skin test or complement-fixation reactions (112). Thus, *Histoplasma* antiserum does not react with *Candida* or *Coccidioides* as antigens, although antisera against the latter react with histoplasmin as the antigen.

The complement-fixation test is useful in the diagnosis of systemic histoplasmosis. Furcolow, Bunnell & Tenenberg (142) found that eight of nine proved cases of histoplasmosis showed a positive complement-fixation reaction, and 10 of 36 sera from nontuberculin reactors with pulmonary lesions who were thought to have benign histoplasmosis, had positive complement-fixation tests. Among 242 normal individuals there were about 5½ per cent who gave positive complement-fixation reactions. These the authors classify as false positives. In this latter study histoplasmin was used as the antigen. Campbell & Saslaw's (168) studies on human sera are not strictly comparable, since they used a ground yeast phase as the antigen. With their method they found a positive complement-fixation reaction in about 35 per cent of normal individuals. Reduction of the incubation period to 4 hr. instead of the previously recommended 18 hr. reduced the false positive reactions to about 6 per cent, a figure which is comparable to that reported by Furcolow and his associates. None of the false positive

reactor group, however, had a titer exceeding 1:20. Nonetheless, the possibility of a false positive reaction must be considered in interpreting the results of the complement-fixation tests in humans. The problem does not invalidate the value of the complement-fixation reaction. The sera of five out of seven proved cases of histoplasmosis were shown to contain complement-fixing antibodies by Campbell & Saslaw. One serum was anti-complementary and could not be used. The titers of those in the acute stage of the disease (3 cases) greatly exceeded those obtained with sera from chronic cases. There was evidence that the high titers fell as the disease entered the chronic phase. A high titer is of considerable diagnostic significance since it evidently implies acute infection. The complement-fixation reaction should probably be repeated at intervals throughout the course of the illness as a possible aid in determining the prognosis. Persistently high titers may presage a fatal outcome. In two of the above acute cases the attempt to isolate *H. capsulatum* was not initiated until the high complement-fixation titer was discovered. The usefulness of this test as an aid to diagnosis in cases of obscure illness is probably considerable.

Skin testing with histoplasmin does not increase the complement-fixing titer (168). There is, however, a somewhat greater incidence of positive complement-fixation reactions in individuals with positive histoplasmin skin test than there is in non-reactors.

Bunnell & Furcolow (133) reported positive complement-fixation antibodies in the sera of nine of their ten proved cases of histoplasmosis. In this group there were frequent cross reactions to blastomycin. A titration of the antisera, however, clearly revealed higher titers with the homologous antigen. As a practical measure the complement-fixation test should be carried out using both histoplasmin and blastomycin as antigens.

The precipitin reaction in animals becomes manifest early in the infection, reaches a peak in three weeks, and becomes negative after about two months. Whereas Scheff (166) failed to find precipitins in the sera of infected animals, he probably erred unknowingly in collecting his sera several months or more after infection. The reaction thus seems to correlate with the acute phase of the disease and needs study in humans.

Anti-*Histoplasma* antibodies may be detected in the sera by means of the collodion agglutination test of Cavelti. Clear-cut endpoints are to be observed with this method, using animal antisera (146, 147). Studies with human antisera are desirable.

It is interesting to note that systemic histoplasmosis, which is essentially a reticulo-endothelial disease with little resemblance to the benign pulmonary form, has not been observed to eventuate directly from dissemination of the pulmonary form. It may not be premature to consider the pulmonary form the primary type of the disease, and to regard the systemic form as the secondary type. This would correspond immuno-pathologically to coccidioidomycosis.

*Coccidioidomycosis and Coccidioides immitis.*—The value of immunologic studies in the investigation of mycotic disease is well illustrated in coccidioidomycosis. By means of extensive skin testing the endemic area for

coccidioidomycosis has been more or less accurately defined (169 to 176). The skin test is of sufficient diagnostic importance so that diagnosis of primary coccidioidomycosis may be considered questionable when the cutaneous reaction is persistently negative. The complement-fixation reaction appears to be helpful in defining prognosis (132). The rapid advances in knowledge of this disease during the last 15 years have been made largely by means of immunologic researches.

Coccidioidin, the material used for skin testing, is a culture filtrate similar in nature to that used in the preparation of tuberculin. The most widely used coccidioidin is that of Smith, produced in a synthetic medium which contains as its nitrogen sources asparagine and ammonium chloride (132). Coccidioidin, however, has been prepared in many different ways (177 to 183).

The first skin testing material was actually a mycelial suspension so that the active principal was present both in the filtrate and the fungus itself (177). The skin testing principle is heat stable and is not destroyed even by autoclaving (169, 181, 183, 184). Concentration of the filtrate to one-tenth volume does not improve its potency (132).

Hirsch & D'Andrea identified the skin testing principal as a polysaccharide (181). Hassid and his colleagues (185), in a more extensive study, showed that the active material is a polysaccharide consisting of galacturonic acid, glucose, and some unidentified sugar. The polysaccharide is associated with a nitrogenous component which is non-protein in nature. Various isolates of *C. immitis* so far studied do not differ significantly in their ability to elaborate the skin testing principle. Autogenous coccidioidins are thus not preferable to stock preparations. But for unknown reasons, some culture filtrates are inferior as skin-testing antigens (132). It is essential, therefore, that some attempt at standardization be made to avoid the use of inactive lots.

Smith and associates find that standardization on sensitized guinea pigs, as suggested by Stewart & Kimura (184), is impractical because of a varying sensitivity (132). They have been compelled to standardize their coccidioidin on human reactors whose sensitivity to a given lot is known.

It would appear that one cannot state the optimal time for harvesting the coccidioidin. It is necessary, therefore, to remove samples of the filtrate beginning at about 16 weeks and to test their potency on humans until a maximum potency is obtained. Smith used ten different strains of *C. immitis* in making up his coccidioidin, but this precaution does not appear to be necessary since strain variations in this respect have not been detected. It may be mentioned here that Salvin & Hottle's (161) method of investigating the potency of a given lot of histoplasmin by determining its capacity to fix complement should be investigated as a possible means of standardizing coccidioidin.

The coccidioidin reaction is of the delayed tuberculin type. Occasionally an immediate urticarial type of reaction is observed, the significance of which is not yet apparent (180, 181, 186). Smith and his colleagues have observed such reactions even with sterile distilled water and are inclined to

regard the immediate reaction as nonspecific. It has not proved possible to transfer the tuberculin-type sensitivity by means of the Prausnitz-Küstner passive transfer test.

Lawrence (187) has recently demonstrated the transfer of tuberculin sensitivity by means of living leucocytes. This reaction should be investigated in the systemic mycoses in which cutaneous sensitivity develops.

Smith *et al.* (132) report that the peak of the cutaneous reactivity is approximately 36 hr. They state that at 24 hr. some slow reactions may be missed, while at 48 hr. some may have waned. Presumably readings should be made at 24 and 48 hr. although for routine epidemiologic purposes either 24 or 48 hr. readings will suffice. For routine surveys a 1:100 dilution of coccidioidin is advised. Approximately 10 per cent of reactors will be missed when 1:1000 coccidioidin is employed (132). With 1:10 coccidioidin reactions may be nonspecific. For hospitalized active cases with primary coccidioidomycosis, 1:1000 is an appropriate dilution (188, 189, 190).

Coccidioidin sensitivity becomes active two days to three weeks after the onset of the disease (175, 191). Within the first week of illness about one-eighth of the cases fail to react (132). After two weeks of illness only a very few patients fail to react. Willett (192) reported a patient who did not become positive for six weeks. It is evident that the coccidioidin test must be repeated before concluding that the reaction is negative.

The severity of the reaction has been observed to increase within the first week or so after becoming positive. The allergic state of the patient with primary coccidioidomycosis may be so marked that a routine cutaneous test precipitates an episode of erythema nodosum (132). Patients with erythema nodosum are uniformly strongly reactive to coccidioidin so that greater dilutions should be used on such patients.

Hirsch & D'Andrea (186) found that animals could be sensitized by injections of killed mycelium. This, however, cannot be accomplished with coccidioidin. It has been conclusively demonstrated that repeated testing does not lead to sensitization in humans nor do circulating antibodies develop as a result of such a procedure (132).

Cohen & Gifford (193) prepared a coccidioidin patch test similar to the Vollmer patch test for tuberculosis. Of 120 patients reacting to intradermal coccidioidin tests, only 22½ per cent gave positive reactions to the patch test. The patch test is evidently of little value. The same is true for histoplasmosis (162).

Cheney & Denenholz (190) reported that cutaneous reactivity to coccidioidin was rapidly lost in some patients. The more extensive experience of Smith (132), however, indicates that this sensitivity persists indefinitely. Occasionally diminution in the reaction may be observed over a period of years.

Dahlstrom (194) reports that of 2,490 tuberculin sensitive individuals who were followed for five to fifteen years, 11 per cent subsequently lost their sensitivity. It is possible that a similar phenomenon may be noted in coccidioidomycosis, particularly in those whose reaction is minimal at the start. By and large, however, sensitivity to coccidioidin is not rapidly lost.

Butt & Hoffman (195) have furnished evidence of the presence of healed calcified lesions in the lungs of coccidioidin reactors at autopsy. In 73 per cent of such lungs *Coccidioides* spherules were present, an anatomic finding which probably accounts for the persistence of the cutaneous reaction.

The problem of cross reactivity in coccidioidomycosis has already been discussed in the sections on histoplasmosis and blastomycosis. Very extensive surveys have revealed that coccidioidin is a relatively specific antigen and the individuals who react to it are by and large residents of the arid southwest.

No cross reaction to coccidioidin is observed in tuberculosis (176, 183). A certain amount of cross reactions occur in individuals who live in the area endemic for histoplasmosis. Such individuals are primarily reactors to histoplasmin who also react in a lesser degree to coccidioidin (136, 196).

The percentage of cross reaction between coccidioidin and histoplasmin appears to be related to the intensity of the cutaneous response to the homologous antigen. For instance, in those individuals with only a 1+ cutaneous sensitivity to histoplasmin, there is only a 20 per cent incidence of cross reaction to coccidioidin. This incidence amounts to 56 per cent in individuals with a 4+ histoplasmin sensitivity. On the other hand, there is practically 100 per cent cross reaction to histoplasmin by coccidioidin reactors whose sensitivity is 2+ or more. Strong coccidioidin reactors also show cross reaction to blastomycin. There is usually little difficulty in recognizing the homologous antigen and, in case a question arises, the antigens may be titrated.

There is strong cross reactivity between haplosporangin and coccidioidin (134). This has been demonstrated both in animals and in humans, but at the present time has little clinical significance.

A curious phenomenon has been observed in respect to the coccidioidin reaction in individuals with the disseminated form of the disease. Dissemination of the disease appears to be associated with a reduced sensitivity to coccidioidin (132, 188, 191, 197, 198). Such reduced sensitivity occurs early in the disease and is not the same as the anergic state seen in the terminal phases of histoplasmosis, blastomycosis and other chronic infections. In fact, the gradual diminution in coccidioidin activity has been observed while dissemination was in progress and the phenomenon appears with such regularity that Smith considers it of prognostic importance. The outlook is more favorable in patients in whom sensitivity is maintained. According to Smith *et al.* (132) three-fourths of the patients who continue to react to, 1:100 coccidioidin will survive, while only one-sixth of those negative to this dilution will recover. Approximately half of those who continue to react to the relatively strong 1:10 dilution will recover; while only one-tenth of the non-reactors to this dilution survive. The possible exception to this general principle exists in patients with meningeal involvement. Thus, repeated coccidioidin tests in the primary phase of infection provides a useful means of estimating the likelihood of dissemination. So far as we are aware this is a unique function for a skin test in infectious disease. On a number of occasions a return of sensitivity to coccidioidin has been noted following recovery

from the progressive phase of the disease. A really valid explanation for the loss of sensitivity during dissemination of the disease is not at hand.

Circulating antibodies appear with great regularity in the sera of those with the disseminated form of the disease (188, 196). These may be determined by the complement-fixation test employing coccidioidin as the antigen. The mechanics of the complement-fixation tests have not been as minutely studied as is the case with histoplasmosis and blastomycosis, or at least such studies have not been reported in detail.

The problem of cross reactivity in the complement-fixation tests has already been discussed. Suffice it to say that cross reactivity with other fungus antigens, such as histoplasmin, is of a very low order and probably has little clinical significance.

The complement-fixation test may become positive during the primary phase of the disease, particularly when the infection is severe. Precipitins also become demonstrable in the early stages and are present in higher dilution than the complement-fixing antibodies (188). However, the precipitins disappear earlier. One notes here a possible similarity to histoplasmosis in which precipitins appear only for a relatively brief period in the acute phase of the disease.

The complement-fixation test has prognostic value in coccidioidomycosis. A rising complement-fixation titer in the early phases of the disease presages dissemination (192, 199). A rising titer may be the first indication of clinical progression. Repeated complement-fixation tests are thus in order for purposes of evaluation of the clinical status of the patient.

The majority of individuals living in an endemic area acquire coccidioidomycosis at some time during their lives, yet the disseminated form of the disease is relatively rare. It is possible, of course, that primary infections establish an acquired immunity to the more severe forms of the disease. Experimental proof of this is as yet lacking.

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# TULAREMIA

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This review covers most published papers from January, 1939 to October, 1949 with excursions into the classical or Francis period of the disease whenever it seemed advisable to relate recent work to the monumental foundations laid by men of the United States Public Health Service.

## Morphology

*Bacterium tularense* (*Pasteurella tularensis*) was described by Ôhara (1) as a motile, monotrichate, encapsulated bacterium, and its pleomorphism was regarded as a consequence of its intrinsic motility. Hesselbrock & Foshay (2) concluded from a systematic study of 42 strains that it was nonmotile and nonencapsulated, that under optimal conditions of cultivation it displayed a constant range of pleomorphism, and that all strains were alike regardless of virulence, age, or source. The terms that described most of its morphologic units are those that describe the morphology of the pleuropneumonia group. In addition, forms suggestive of sporangia and sporangiospores were seen, and the usual coccoid form was shown to be a spheroidal cystic structure with transparent, delicate cell wall and peripherally aggregated chromatin masses that arise from the cell wall and project into the cavity which seems to contain a fluid of low viscosity. The morphology and modes of reproduction, chiefly by budding, suggest relationships with fungi and the pleuropneumonia group and none whatever with *Pasteurella* or *Brucella*. Discrete units as small as 300 to 350 mμ. were demonstrated by ultrafiltration (3). Such units produced tularemia in mice, and cultures from the mice again showed the complete gamut of morphologic units. The essential features of this study were confirmed by the electron micrographic studies of Eigelsbach *et al.* (4) which showed that most units had extremely delicate, almost electron transparent cell walls unlike those of any known bacterium, a feature that may explain the organism's ease of sonic disruption and its lyophilization instability. Discrete units as small as 110 to 210 mμ. were observed.

## Cultivation

Ransmeier & Stekol (5) confirmed the obligate cystine or cysteine requirement and stated that of other sulfur-containing compounds tried in serum infusion agar, only glutathione and thioglycollic acid would promote growth. Ransmeier & Schaub (6) found that "sodium thioglycollate may be substituted for the cystine in blood glucose cystine agar and will support the growth of *B. tularense*." Snyder *et al.* (7) also say in relation to their peptone broth that "cystine may be replaced by 0.01 per cent thioglycollic acid."

Tamura & Gibby (8), who pioneered a great advance by developing the first liquid media, confirmed the utilization of glutathione but showed that when all cystine was excluded, growth could be neither initiated nor supported by thioglycollate. Their medium permitted sustained growth from inocula of 1 to 15 cells per ml. of medium. No toxin was produced by luxuriant growth up to 14 days, but alkali was formed in liquid cultures.

The simplest liquid medium for large scale cultivation is the peptone broth of Snyder *et al.* (9). Their paper gives an excellent study of growth conditions. The medium was improved by their addition of cysteine (7), and solidification of it with agar produced a plating medium of value for dilution counts. Larson (10) found no medium to be as delicate a detector as mouse inoculation though his hydrolysate medium was obviously not up to standard, and his modification of the Steinhaus (11) medium may not have been fully exploited. Gibby *et al.* (12) added an extract of blood cells to protein hydrolysates, creating a clear liquid medium capable of massive cell yields from large inocula and of sustained growth from 1 to 5 cells per ml. of medium. The extract contained undetermined substances that permitted a metabolic stabilization of the pH despite concurrent and irregular production of acid and base in an unbuffered medium.

Johnson (13) observed continued multiplication in minced nine-day chick embryo tissue in serum Tyrode solution with transfers every three or four days. Mouse virulence was maintained through 31 serial subcultivations. In the first chick embryo studies, Buddingh & Womack (14) noted that the preferred ectodermal areas of colonization were intracellular and wherever erythrocytes were present, or at the periphery of focal areas of reaction. Although phagocytosis was not observed, the bacteria disappeared rapidly from purulent exudates.

Ransmeier (15) and Larson (16) each found the chick embryo and white mouse to be about equally susceptible, and Larson noted that the degree of multiplication in embryos was of the order of that within mouse tissues and that considerable multiplication took place in the yolk sac. Downs *et al.* (17) introduced a valuable dilution plate count technique that showed vastly greater multiplication in the yolk sac than elsewhere. They found embryos more susceptible than mice to strains of lowered virulence and noted that even the avirulent 38 strain killed a few. Hence embryo $LD_{50}$ doses are not always equivalent to mouse $LD_{50}$ doses. Miller (18) found that frozen-dried cultures in a mixture of beef infusion, cystine, and cooked rabbit's blood were preserved for 3 years, 10 months.

## Nutrition, Metabolism

Mills *et al.* (19) found that the essential amino acids that replaced hydrolyzed vitamin-free casein in a complete medium containing cystine were methionine, threonine, serine, valine, leucine, isoleucine, tyrosine, histidine, lysine, arginine, proline, aspartic acid, and glutamic acid. O'Kane (20), using a hydrolyzed vitamin-free casein medium, found thiamine to be the

essential heat- and alkali-labile fraction in blood cell extract. Since Tamura & Fleming (21) found that the addition of thiamine to a synthetic medium deficient only in adenosinetriphosphate (ATP) did not permit growth, and they confirmed that thiamine added to gelatin and casein hydrolysate media did initiate growth; the function of thiamine remains unclarified. In the synthetic medium containing ATP or some other source of phosphorus, growth occurred without thiamine. Thus, the importance of priming an otherwise adequate medium with a precursor for phosphorylation is indicated.

## Pathology

Additions to the standard reference monograph on pathology (22) were made by Lillie & Larson's description (23) of the pathology in the golden hamster, and by Ashburn & Miller's report (24) of tissue changes in an early death prior to the end of the fifth day in which polynuclears infiltrated the lungs in greater than usual proportion, caseous necrosis was absent, and intracellular colonizations of *B. tularense* were found, the latter being a pathological rarity. Wells & Tillman (25) recorded the first case of spontaneous rupture of the human spleen on the 13th day of disease with the diagnosis verified by cultures. This recalls Camp's unpublished case (26) of a hunter who, while hunting deer during the third week of a rabbit-borne tularemia infection, slipped and fell across a log and ruptured his spleen. Death occurred 4 hr. after operation on the next day. Sections and cultures of the spleen confirmed the diagnosis.

## Susceptibility and Active Immunity

Larson (27) reported that white rats were about 1,000-fold more resistant than white mice and that culture vaccines, yolk sac, and ether-extracted yolk sac vaccines greatly increased their resistance. The hamster was as susceptible as the white mouse (28). A quantitative study with uniform procedures by Downs *et al.* (29) revealed the order of susceptibility to be mice, hamsters, guinea pigs, rabbits, cotton rats, monkeys, rats, dogs, and 9-day chicks. Downs, Coriell *et al.* (30) verified the vaccine protection of rats by various vaccines against 1,000 $LD_{50}$ but showed that recovered rats resisted rechallenge with 10,000 to 1 million $LD_{50}$. Coriell, King & Smith (31) found the monkey's disease, pathology, and resistance much like those of man and showed that Foshay's phenolized hydrolysate culture prophylactic vaccine protected efficiently against small challenges. Vaccinated Swiss mice were found by Coriell *et al.* (32) to develop slight resistance to small challenges, and rechallenge showed this to be very slight although 30 per cent of healthy survivors harbored highly virulent organisms in the spleen for a month. Ruchman & Foshay (33) used a human prophylactic vaccine in white mice but saw no survivors when they were challenged at the time of peak agglutinin response.

Gotschlich *et al.* (34) first noted the superior immunizing properties of living strains of low virulence in comparison with killed virulent strain

vaccines and were able to protect both guniea pigs and mice against challenges with a virulent strain. They noted retention of the organism in the spleens of resistant animals and called the condition a premunition immunity. Vereninova *et al.* (35) confirmed that avirulent living bacteria protected guinea pigs for about 3 months against a virulent challenge whereas no killed vaccine did so. Larson (27) and Downs (30) found the avirulent strain 38 highly effective in immunizing rats. Downs & Woodward (36) found that sublethal challenge with strains of lower virulence protected mice against 1,000 $LD_{50}$ of two highly virulent American strains, that virulence and immunogenicity of strains could vary independently, and that the pattern of multiplication of low virulent strains in normal mice paralleled that of virulent strains in immunized mice. Killed vaccines and a filtered sonic lysate gave scarcely any protection. Mouse resistance rose to effective levels on the third day after injection of the low virulent strain. The avirulent strain 38 did not protect, and agglutinins provoked by it disappeared in a week.

## Passive Immunity

Francis & Felton (37) could not protect mice against 1 MLD of a virulent strain with antiserums or human convalescent serum and concluded that their method failed to show protective antibody, implying that serum therapy of man would be useless. Bell & Kahn (38) failed to protect guinea pigs with antiserum or with many chemical agents and concluded that clinical use of antiserum would be ineffective. Foshay, Ruchman & Nicholes (39) showed that the white rat which is naturally resistant, like man, could be protected against 25,000 $LD_{50}$ with various antiserums, that protective antibody was correlated with polysaccharide precipitable antibody, and that results from naturally nonresistant test animals have no meaning with respect to either vaccine prophylaxis or serum therapy of man. Larson's rat studies were confirmatory though too little antibody was given to obtain constant results (40). The results of serum therapy in man were comparable in many ways to those in the rat (41). The protective property, for mice of antiserum prepared in horses, of up to 80 per cent as reported by Vereninova *et al.* (42) possibly reflects either the lower intrinsic virulence of Russian strains or a more resistant strain of mice.

## Pathogenesis

Downs *et al.* (43) showed that *B. tularense* invaded rapidly all rats but multiplied less in the vaccinated and still less in the recovered rat, with systemic invasion delayed in the vaccinated and recovered immune rats. The latter resisted more effectively than did vaccinated rats. Buchele & Downs (44) found that vaccination caused a rapid rise in resistance, highly effective by the second day after vaccination and that light infections left recovered rats as solidly immune as heavy infections. Although bacteria were not recoverable from the spleens of recovered rats for longer than one month, the rats had agglutinin titers of 1:100 and were solidly resistant.

## Immunology

Larson (45) found in formalinized cell suspensions a heat stable antigenic fraction, unrelated to virulence, that caused characteristic dermoreactions in rabbits which could be neutralized with antiserum and partially inhibited by previous vaccination. He also applied the Ascoli thermoprecipitation test to tularemic materials, a test of value in earlier diagnosis and in rodent survey detection (46). Downs, Coriell *et al.* (30) in preliminary fractionation studies characterized several crude antigenic fractions and found immunizing properties in a protein and a protein-carbohydrate fraction. Morey & Spies (47) found that persons with malnutrition and slight vitamin deficiencies reacted to tularemic vaccine with normal agglutinin curves. Persons with moderate deficiency states produced lower peak titers and curves of one-sixth the normal duration. People with severe pellagra produced very low titers that disappeared rapidly and which were of about one-fortieth the normal duration.

## Experimental Chemotherapy

Ransmeier (48) and Tamura (49) agreed that experimental sulfonamide therapy failed of practical, useful effect in chick embryos and in culture media. The latter found that the usual quantitative inhibition by *p*-aminobenzoic acid (PAB) did not occur with sulfapyrazine.

The studies of Heilman (50), Tamura & Suyemoto (51) and of Chapman *et al.* (52, 53, 54) leave no doubt that streptomycin is both bacteriostatic and bactericidal *in vitro* and is a highly effective agent in the treatment of tularemia in the white mouse, white rat, and monkey. In Tamura's test, the $ED_{50}$ dose of streptomycin against a 50 $LD_{50}$ challenge was 350 $\mu$g. per 20 gm. mouse, or 17.5 $\mu$g. per gm. None of Chapman's or Tamura's recovered mice were immune to rechallenge, though 30 per cent of Chapman's mice harbored virulent organisms in the spleen for at least 50 days. Recovered rats, rechallenged, showed that the longer the interval between initial infection and therapy the more solid was the resistance. Monkey tularemia showed many likenesses to the human disease, including response to streptomycin.

Aureomycin studies in mice were designed by Woodward *et al.* (55) to compare its efficacy with that of streptomycin and chloramphenicol. In the only mouse series that gave survivals, the dosage of aureomycin was 30 per cent greater than that of the other agents. Ransmeier (56) found aureomycin to have a bacteriostatic effect in infected mice, but it spared scarcely a mouse owing to too short a treatment period. Eigelsbach & Herring (57) determined that the *in vitro* bacteriostatic level against $10^6$ cells per ml. in a suitable medium was 2.5 $\mu$g. per ml. for aureomycin and 2.0 $\mu$g. per ml. for chloramphenicol. They found each of these antibiotics capable of sparing mice challenged with 100 $LD_{50}$, each proving to be only bacteriostatic *in vivo;* administration of either drug for 10 days gave temporary suppression but total deaths after cessation of administration. The same dosages of either drug for 20 days permitted survival of most mice. The inference suggested by Woodward *et al.* (55, 58) that chloramphenicol is inferior to aureomycin

and that it might be a less effective agent for the human disease, does not follow from the inadequate experimental work presented.

## Vaccine Prophylaxis

The initial prophylactic vaccine of Foshay *et al.* (59) and its improved phenolized liquid culture successor have protected many exposed persons and have modified significantly the course of disease of those who acquired it (60), notably among laboratory personnel. A report of the first trial with Gaisky's living avirulent vaccine on 46 women and 4 men is given by Kosmachevsky (61). About half took 5,000 cells of a strain "virulent for mice but avirulent for guinea pigs" and the other half took from 5,000 to 500,000 cells of strains with "minimal virulence for mice," all doses given in 1 cc. amounts. Local and general reactions occurred in 36 cases (72 per cent) with prolonged moderate disability in some and fairly severe disability in a few.

## Laboratory Infections

Reports by Ducey (62), Ashburn & Miller (63), and Jordan & Downs (64) reemphasize the long known laboratory risk of this disease, and one fatality indicates a broadening need for prophylaxis among laboratory personnel. During four years of work on tularemia at the Central Institute of Hygiene at Ankara, there occurred four laboratory infections, of which two were totally inapparent, discovered only by routine agglutination tests according to Golem (65). These are the first inapparent tularemic infections to be reported.

## Laboratory Diagnosis

Slide agglutination tests for rapid bedside diagnosis were found to be useful by Muraviev & Ivanova (66), Korth (67), and Tovar (68) providing the serum titer was not below 1:20. Johnson (69) indicated the diagnostic value of cultures and animal inoculations of nasal secretions, calling attention to this little considered mode of exit from the body, and Larson demonstrated *B. tularense* in pharyngeal secretions of patients without pneumonia (70). Damon & Johnson (71) and Nagle *et al.* (72) found that the macroscopic serum agglutination procedure would give immediate results if the tubes were shaken vigorously for 5 or 6 min. providing the titers were not too low, an observation first recorded by McClanahan (73). Tovar (74) found that 50 per cent of brucellosis sera and 37 per cent of tularemia sera cross agglutinated and also noted cross opsonification and cross dermoallergic responses. These latter, with cross agglutinations at low homologous titers, suggest anamnestic responses in unapparent or chronic brucellosis rather than true cross reactions. Friedewald & Hunt (75) studied the noncultural tests in 50 patients and found the intradermal test to be highly reliable, provoking such slight agglutinin formation (1:20 to 1:40) in but one-third of normal persons after two weeks that confusion with the agglutinin curve of the disease was most unlikely. Drobinsky (76) confirmed the specificity and reliability of the

intradermal test, finding it positive in 75 per cent of cases during the first week of disease and in 95 per cent of cases thereafter. He found that nonspecific reactions were rare in tularemia and that cross reactions with brucella antigens "were insignificant." Ransmeier & Ewing (77) reviewed knowledge of the agglutination test and published a useful composite curve. They cited one case of highly presumptive but unproven tularemia in which serum agglutinins had disappeared after 3.5 years. Pulaski & Amspacher (78) also cited a similar event in another unverified case following streptomycin therapy. If the diagnosis were correct, this is the first instance of agglutinin loss after such therapy. Lawless (79) prepared a Frei type of antigen from bubo pus which provoked dermoreactions in patients and in recovered immunes, but not in normal persons. This antigen agglutinated the erythrocytes of infected persons and provoked a histiocytic response in dermal tissues. Vignati & Šantavý (80) noted a two-fold increase in both the reduced and oxidized forms of blood glutathione in 15 patients.

Sugar fermentations by Francis (81) on solid media with bromthymol blue as indicator showed that all of 60 strains produced acid from glucose, maltose, and mannose; 53 produced acid from glycerol; 17 from levulose; and 30 produced acid from dextrin. No other sugars gave acid reactions. Golem (82), also using solid media, found Turkish strains produced acid from glucose, levulose, dulcitol, arabinose, and galactose, but not from glycerol, dextrin, or others. Francis observed that "in isolated instances the reaction swung to the alkaline side." Gibby *et al.* (12) noted that some cultures in favorable liquid media formed alkali so rapidly that sugar utilization occurred in media that became progressively alkaline and that changes in pH were not reliable indicators of carbohydrate utilization.

## Clinical

Excellent clinical reviews were written by Blackford & Casey (83), Pullen & Stuart (84, 85), Foshay (41), and Francis (86), the latter notable for its clarity of distinctions between tularemia and Parinaud's syndrome and between scientific and inspired methods of diagnosis. Heart and pericardial involvements were discussed by Aagaard (87) and by Jager & Ransmeier (88). Meningitis and meningeal involvement were studied by David & Owens (89) and Glass (90, 91), and an extraordinary case complicated by trauma was reported by Fields (92), whose paper is also valuable for the extended reference to the experiences of Drobinsky. Stuart & Pullen's case was unusually atypical and unfortunately not completely established as tularemic (93).

Frank & Wohlfeil (94) described 43 definite and 8 possible cases of mild chronic tularemia among Russian workers in civilian camps in Germany, all apparently relapses or recrudescences of infections acquired in Russia, yet *B. tularense* was isolated from the lymph nodes of three of them. The low agglutinin titers, usually 1:5 to 1:40, may represent transitory agglutinin absorptions consequent upon endogenous reinfection. It is a type of disease

rarely seen in this country but one that might become more frequent if vaccine prophylaxis became widespread. Lide (95) reported the first example of congenital transmission with death of the fetus, the infection being acquired during the eighth month of pregnancy.

## Therapy

The collected experience of 490 clinicians with serum therapy, reported by Foshay (41), showed that as a moderately good bacteriostatic agent serum reduced mortality and shortened morbidity. Vaccine therapy was tried by Bilibin (96) and found to be effective but slow, tedious, and requiring skill beyond the ordinary to avoid general reactions. It was abandoned for the same reasons that caused the reviewer to discontinue it (97). Jackson (98) stated that bismuth sodium tartrate was a specific remedy, a conclusion which the quantitative data that he cited for three illustrative cases do not support, but one that is supported by the statement of Monnet (99). Although the report of Ecke & Ecke (100) lacks some essentials, the apparent efficacy of atabrine therapy deserves further study. Streptomycin has become a universally recognized therapeutic agent of high efficacy, so efficient that in the presence of large exudates, treatment provokes analogues of the Herxheimer reaction, most of which are misunderstood or misinterpreted. The bibliography is already large, and it seems adequate to document the above statements by selected references to the papers of Foshay & Pasternack (101), Cohen & Lasser (102), Howe, Coriell *et al.* (103), Hunt (104), Morgan (105), Foshay (106, 107), and Berson & Harwell (108).

Aureomycin in small initial groups studied by Woodward *et al.* (55, 58) and by Ransmeier, Price & Barnes (109) appeared to be effective, though the statement by the former group that its degree of efficacy is comparable to that of streptomycin is not supported by the dosages used.

## Epidemiology, Sources of Infection

Oosting (110) reported an ocular infection acquired from a tree squirrel, a rarity in Ohio. The second case of infection from handling pheasants was recorded by Kursban & Foshay (111). Since the Public Health Service reported 13 human cases from cats in 1940 (112), seven new cases from the same source were reported by Jungherr (113), Shaffer (114), and Siniscal (115). Falk (116) and Miller (117) each reported mechanical transfer infections from handling freshly caught fish, each implicating river water as the source of the infection. Muskrat-borne infections occurred in New York (118) and Indiana; the latter also reported cases from squirrels, opossum, and cat (119).

Natural infections were noted in dogs by Ey & Daniels (120) and by Johnson (121), whose excellent paper includes studies on susceptibility, transmission, and pathogenesis in dogs, cats, and chicks. Parker verified natural infection of the chipmunk (122), and the first infected shrew was found by Kohls & Steinhaus (123).

## New Geographic Areas

The first human case in Alaska, apparently contracted from muskrats, was reported by Williams (124), and the first in Connecticut by Gibbons *et al.* (125), leaving Vermont the only state without reported cases. The first human case in Mexico was verified culturally by Tovar (126), who found by serum agglutination survey that tularemia existed in the states of Michoacan, Guanajuato, Jalisco, Mexico, Tlaxcala, and Guerrero, all within the tropics, with rabbits and ticks as the chief sources of infection. Of 149 dog sera, Varela & Mariotte found 3.4 per cent to agglutinate from 1:40 to 1:1,280, but of 1,166 rabbit sera, none agglutinated (127). Schoop (128) verified natural infection in hares in western Poland, and Direk (129) extended the known range in Turkey eastward to the Lake Van area. Golem (130) found that experimentally infected frogs contaminated water which infected fresh frogs, some establishing latent infections and excreting virulent organisms back into the water. Brook water brought into the laboratory was infective for mice for 25 days. Water buffaloes were found to be susceptible by many routes, but none died. All excreted virulent organisms in the urine for at least 37 days, establishing another source for natural water contamination. A serum agglutination survey recorded more positive titers from women than from men and also revealed an unsolved transmission puzzle involving areas with positive sera from man and buffaloes but none from horses and other areas with 58 per cent of horse sera positive but none from man (131). Land turtles were found to be susceptible but resistant, developing latent infections that persisted for 45 days with excreta infective for guinea pigs and contaminative for water. *Hyalomma aegyptium* became infected from turtles, but natural transmission of the disease by the ticks was not established (138).

France saw human cases first verified in 1946 (132), a third proven case by wild boar bite in 1947 (133), and a summary of 18 cases by Girard before the end of that year (134). Two verified cases from hare contacts occurred thereafter near Sedan (135), also a tick-borne infection in Haute Marne, apparently from *Dermacentor marginata* (136). The total number of French cases was 33 by September, 1949 (99). Although a dead hare in Italy yielded a pure culture of *B. tularense* in 1931, no human case has yet been reported from that country (137).

## Modes of Transmission

Whereas Washburn & Tuohy (139) and Bost *et al.* (140) found the disease to be chiefly tick-borne in Arkansas, and Rosson found it of rare occurrence in Tulare County (141), the papers of Jellison & Parker (142), Morgan (143), and Bow & Brown (144) incriminate rabbits, especially the cottontail, as the chief North American transmitter to man. In the United States and Canada the areas of prevalence coincide sharply with the range limits of *Sylvilagus*. The varying hare, or snowshoe rabbit, is insignificant as a transmitter to man but, together with many small mammals and rodents and their ectoparasites, it serves to maintain a natural reservoir of infection al-

though its high natural resistance, carrier state, and acquired immunity serve to modify greatly that infection with respect to virulence and transmissibility. The admirable studies of Green (145, 146, 147) supported by those of Davis & Philip (148, 149) revealed the biologic importance of the varying hare and of certain gallinaceous birds to *B. tularense*, diminishing its virulence and limiting its transfer to *Haemaphysalis*, the rabbit tick that is the chief transmitter among rabbits and birds, and even aiding in clearing infection from ticks previously infected. Green thought that the uniformity of the typhoidal type of disease among American laboratory workers represented the uniformity of virulence of a guinea pig adapted strain (146). The importance of immune hosts to epizootiology in limiting tick transmissibility was noted by Bell (150) for *Dermacentor variabilis*, a tick in which hereditary transmission was found to be the exception rather than the rule.

## Arthropod Transmission

Burroughs *et al.* (151) found that meadow mouse fleas were poor transmitters and that these mice harbored latent infections in their spleens; they gave a useful list of known naturally infected vertebrates. Prince & McMahon (152) found that the rat flea, *Xenopsylla cheopis*, and the ground squirrel flea, *Diamanus montanus*, both efficient transmitters of plague, were poor transmitters of tularemia, but flea feces were infective for at least 3 weeks after drying. Volfertz & Kolpakova (153) and Olsufiev (154) found that rodent fleas were poor transmitters but that ground squirrels became chronically infected carriers, a fact also noted in Russia by Gorokhov & Kasantsera (155) and in Canada by Brown & Roy (156) and Humphries & Campbell (157).

Davis (158) noted that *Cimex lectularius* failed to transmit infection to guinea pigs if contamination of the animals by bug feces was prevented and also that transovarial passage did not occur, either by feeding or by injection; these results are in harmony with all American experience but in conflict with those of Bogenko (159), Kamil & Bilal (160) and Bilal [in Golem (82)] in Eastern Europe.

Mosquito transmission to man occurred in Sweden where hares and lemmings form the natural reservoir. Olin (161) demonstrated natural infection in *Aedes cinereus* in an edemic area and later reported that of 548 cases during a seven-year period, only 18 were attributable to animal contacts (162). Thjøtta's case indicated mosquito transmission in Norway (163), as did the studies of Glass in Siberia (90, 91). Karpov *et al.* (164) isolated *B. tularense* from *A. cinereus* and *A. excrucians*, and Maisky (165) reported summer mosquito-borne epidemics in Russia.

Tick transmission maintains and perpetuates the natural reservoirs among lagomorphs and rodents. *Ornithodoros lahorensis*, widespread in Turkey, was found by Bilal (166) to be an important vector. Detailed studies of ticks important in epizootiology in Russia were reported by Karpov & Popov (167) and Olsufiev (154), the chief ones being *Ixodes ricinus*, *I. persulcatus*, and *D. pictus*, the last bearing the same relation to field voles

that *Haemaphysalis leporis-palustris* does to American rabbits. Tick-borne epidemics in Tennessee were reported by Byfield *et al.* (168) and by Warring & Ruffin (169), apparently caused by *Amblyomma americanum*.

The folly of importing wild rabbits from known regions of endemicity into a tick ridden area was exemplified in southeastern Massachusetts, where prior to 1937, no cases had occurred and, because of the recognized tick problem, physicians (170) had urged that such importation be prohibited. Badger (171) reported the first human case three months after importation of the first of 7,000 rabbits and the second case, by tick bite, occurred a year later (173), with a third, also by tick bite, near the same place in the following year (172). Belding (173) verified tularemia in incoming rabbits from Missouri and Arkansas on two occasions in 1940. Moore *et al.* (174) reported a fourth case by tick bite from the same area and another near Boston also apparently tick-borne, bringing the New England total up to 20 cases by 1944. Greer (175) reported another tick bite case from the original importation focus, one of seven reported for the state in 1947, and Ayres & Feemster (176) reported four additional tick bite cases from near the original area, an established focus by 1948, and warned that since no satisfactory control measures for the tick population had been devised, the increasing use of personal prophylaxis had become imperative. It might be noted here that although the varying hare is abundant in northern New England, this region is conspicuous for its low incidence of tularemia.

In Central Asia, Olsufiev (177) found that tabanid flies were important in maintaining the natural vertebrate reservoir and that flies infected by contaminated water were infective by biting for 3 weeks at 20 to 21°C. Excellent papers on epidemiology were published by Tartakow (178) and by Ayres & Feemster (176).

## Epidemics of Murine Origin

Puntigam (179) described two outbreaks in Austria that followed antecedent plagues among field mice; in each there were about 200 cases, and there was one death in an exhausted returned prisoner of war.

Tularemia became a military hazard of prime importance during the late war in Europe. Maisky (180) relates that during the early years, when the harvest could not be gathered, there occurred a great multiplication of small rodents, chiefly the field voles, and that epizootics occurred in 11 of 18 species of other South Russian mammals. He (165) and Apekhtin (181) described four types of epidemic outbreaks in man: (*a*) occupational, bubonic, from hunting water voles, in early spring; (*b*) insect-borne, chiefly bubonic, in summer; (*c*) massive outbreaks, anginal type, water-borne; (*d*) postepizootic, typhoidal, pneumonic, and influenzal types, infection by inhalation from threshing or from house mice feces in dusts in homes or among troops in trenches, in autumn and early winter. According to Khatenever (182), the chief transmission cycle is water rat to field mouse to house mouse to man, and infection by ingestion is also frequent. Among symptoms described by him in an inhalation outbreak, cough, thoracic pain, and evidences of pneu-

monia were minimal. The fatality rate was 0.85 per cent, and inapparent infections were detected. Rudniev (183) found dust-borne infections more prevalent than droplet-borne, and noted that inhalation infections were characterized clinically by prevalence of the influenzal or upper respiratory type, with mediastinal lymphadenopathy and prolonged harassing cough, rather than the pneumonic type.

The papers of Schad (184), Schuller & Erdmann (185), Schulten & Scheppach (186) and Milkat & Kuhlmann (187) tell a similar story of murine outbreaks in the German army, one with 80 per cent of cases of the typhoidal type, thoracic form, with bronchitis, mediastinal lymphadenopathy, and inconsiderable pneumonic form.

## Water-Borne Epidemics

Golem (188) described a small water-borne outbreak in Thrace, at the site of an earlier one eight years previously. The cases were characterized by extreme mildness. All were either glandular or oculoglandular in type, and four wholly inapparent cases were discovered by serum agglutination tests. No new cases occurred after use of the creek water was forbidden. Tsareva (189) reported an urban outbreak that followed diversion of river water into an artesian well distributing system that had been destroyed by enemy action. New cases ceased after chlorination of the water. Following the breakdown of public health and sanitation owing to the war, Schmidt (190) says a great extension of tularemia occurred in southeastern Europe, in which cases were numbered in hundreds of thousands, almost all persons acquiring it in some districts. In the Rostov area alone, there were 8,500 cases in November 1941 and 14,000 in January 1942. The epidemic was water-borne, owing to contamination of wells and streams by water rats. Schmidt states that it was a serious cause of disability in the Russian army.

In agreement with the report of Tsareva (189), Foot *et al.* (191) and Gotovskaia & Mogaram (192) determined that chlorination of waters at about the usual concentrations rendered them safely potable.

## Tularemia in American Waters

In association with an epizootic among beavers, and a probable antecedent outbreak in field mice, Jellison *et al.* (193) verified tularemic contamination of four widely separated streams in Montana. The organism survived in water samples for 16 days, in mud samples for 31 days, and remained virulent in beaver urine for 31 days at 38° to 40°F. Beaver deaths were greatest in still ponds, least in running streams, suggesting the operation of a critical concentration threshold. Further study by Parker *et al.* (194) revealed an extensive river contamination, up to 35 miles at times, and for 16 months in duration, of rivers in Montana, Idaho, and Wyoming. During much of that time no animals of any kind could be found. Frog's tissues did not yield the organism, but snails and fairy shrimps did, but no finding seemed to account for the infectivity of any 10 cc. sample of water over a period of 16 months, with lesser infectivity during the warmer months. Mud

stored in the cold remained infective for 12 weeks, and mud was shown to contain cystine. They speculated about possible survival, or even growth, in mud and in water, a possibility that Gibby *et al.* (12) showed was nutritionally feasible. No human disease attributable with certainty to stream water occurred, although three cases, all with primary angina, occurred in persons who drank stagnant reservoir water.

Parker (195) saw beavers recolonize in contaminated water, raise families, and thrive; people swam in contaminated waters and also thrived, surely curious phenomena for an organism which, under other conditions, penetrates easily the unbroken skin and produces severe illness.

Uncritical writing about primary tularemic pneumonia is recurrent in clinical literature. The use of available data permits the conservative estimate that 3,500 cases of tularemic pneumonia have occurred in the United States since 1925. *B. tularense* is easily recoverable by nasal and pharyngeal swabs, or from sputum, from cases with pneumonia, and from many without pneumonia. Bedside attendants are exposed to droplet infection. If primary, air-borne, tularemic pneumonia exists, some of the attendants of our 3,500 pneumonic cases should have acquired the disease. No secondary case has ever been recorded. In the reviewer's experience with laboratory workers exposed to infectious aerosols and dusts containing American strains of maximal virulence, over 30 individuals acquired tularemia but only two developed small areas of pneumonia, a frequency for pneumonia less than one-half that of the usual rabbit transmitted ulceroglandular type. Primary tularemic pneumonia is found in the clinical literature, but no verified case has yet been found in bed. Johnson (121) observed that the organism was excreted in the nasal secretions of infected cats and dogs, as others have found with other animals, but neither he nor they, by crowding susceptible normal animals into the cages of the ill, saw a normal animal come down. The ways of *B. tularense* in fluid media are mysterious, and something more than analogy with other diseases will be required to elucidate them.

Upon the occasion of the Fifth Pacific Scientific Congress the late R. R. Parker said, "I know of no other infection of animals communicable to man that can be acquired from sources so numerous and so diverse. In short, one can but feel that the status of tularemia, both as a disease in nature and of man, is one of potentiality" (196). After reading the thorough 1939 study by Jusatz (197) of the epidemiology and geography of the disease in Europe, Greenwood (198) commented, "One wonders whether we have to do with an epidemiological curiosity, *viz.*, a disease sharply delimited and only able to exist under conditions which preclude it from becoming more than a curiosity, or whether this is the early stage of something very important. Available evidence favours the former alternative." It seems now that some of the potentialities visualized by Parker 15 years ago have been realized. Nevertheless, it appears unlikely that the disease will assume first rank importance unless the evolving parasitic relationship should include successful adaptation to an effective man to man transmission.

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# BRUCELLOSIS

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The space allotted for this review prevents us from including many subjects of interest which have appeared in the literature during the last two years. The topics considered are those which deserve particular attention for a better understanding of the recognition, pathogenesis, pathology and treatment of human brucellosis. For further information the reader is referred to the discussion of various chapters on Brucellosis presented at the Symposium held in Washington, September, 1949 (20) which it is expected will be published in the near future. Some of the manuscripts were made available through the courtesy of their authors and have been of considerable value in the selection of material for the present review.

## Brucella and the Host

Although accurate data concerning the epidemiologic status of brucellosis are not available, one may consider as valid the general belief that this disease has increased in incidence throughout the world in recent years. In some countries it is consequent to the spread of infection among domestic animals, in others it is the association of cross-infection in cattle with *Brucella melitensis* and *B. suis* which resulted in a new source of these dangerous brucellae (22, 48 to 51, 61).

Little has been added to previous knowledge concerning the means of transmission of *Brucella* from animal to man or from man to man. In the latter case, however, there is evidence that transmission may be by means of the seminal fluid when intercourse takes place during, or soon after, an attack of brucellosis involving the testicle (24, 53). The occurrence of airborne infection, whether from contaminated dust of stables or from the laboratory has been supported by the experimental work of Elberg & Henderson (31).

Once acquired, *B. melitensis* and *B. suis* are likely to produce a rather severe disease, while *B. abortus* has a tendency to cause milder and even unapparent infections. No one questions, however, that severe and fatal cases have been caused by *B. abortus*. Evidence for this has been added by Spink *et al.* (88), and by Anderson (1), the latter describing an important milk-borne epidemic. Experimental work in man (67) and the reports from South America (70, 75, 77) as well as older data from Mediterranean countries, however, fail to duplicate the frequent findings reported in the northern countries. The contrast, perhaps, with the severity of melitensis infections has even resulted in discussion of the possibility of using living cells of *B. abortus* as a vaccine in a desperate attempt to protect man against the

caprine infection (71). The differences in pathogenicity for man of *B. abortus* as observed in well-defined regions is worthy of further investigation.

## Bacteriology

The work of Meyer and co-workers and that of Huddleson and his school continues to be fundamental in the study of *Brucella*. New findings of importance are, however, those concerning the growth requirements of these organisms. It appears that a proper balance of salts, vitamins, and other organic substances, (for example, tryptose with thiamine, glucose and iron salts) increases the yield of the cultures to a considerable degree (62). Gerhard & Wilson (34), have found that ammonium salts can be the sole source of nitrogen for *Brucella*, but that asparagine, glycerol, and lactate provide excellent material and energy for the multiplication of this organism.

The biochemical methods used for the classification of *Brucella* remain the same, although Meyer (65) has insisted that carbon dioxide requirements, hydrogen sulfide production, and the bacteriostatic tests are not entirely safe without proper serologic corroboration. To facilitate the latter cultural test, the use of strips of filter paper impregnated in the dye has been proposed; these are placed over the inoculated agar plate (21). The bacteriostatic test has been applied to the differentiation of ordinary *B. abortus* and strain 19, the latter being more sensitive to thionine-blue (55). In the classification of several thousand recently isolated strains of *Brucella*, the above-mentioned tests have been successfully used and corroborated by selective agglutination (11), a rapid test which gives an accurate determination of the type of agglutinogen.

The reader is referred to the excellent reviews on immunochemistry of *Brucella* recently presented by Elberg (30) and Pennell (72). To this we may add the application in immunology of a method of biochemical analysis used extensively. Jones *et al.* (47) have found, by means of paper-partition chromatography, differences in the amino acid content of normal- and brucella-immune serum. Besides providing additional information on the composition of antibodies, this method may open new fields to serologic diagnosis. Based on a different principle but performed on filter paper submitted to development by capillary absorption is "surface fixation," a new method of demonstrating antigen-antibody reactions with *Brucella* and antiserum (12).

Studies on bacterial variation have been continued by Huddleson (44, 46) who has found that certain mucoid phases of growth of *Brucella* become deprived of pathogenicity but retain their immunizing properties. This author has undertaken extensive application of his findings in the prevention of infectious abortion. Little is known concerning the cause of the variation. Related to this subject is the interesting work of Braun (5, 6); this author has shown that the serum of normal animals, known to be susceptible to brucellosis, has the property of preventing dissociation, a property lacking in the serum of infected animals. The author finds certain differences in the dissociation—inhibiting activity between the sera of infected and vaccinated

animals which he considers of diagnostic value. These interesting phenomena were not observed in similar studies with relatively nonsensitive animals.

## Laboratory Diagnosis

Whatever criticism may be levelled against bacteriological and immunological methods of diagnosis in brucella infection, it should be kept in mind that they are the only possible means for the adequate recognition of such disease. It is evident, however, that faulty technique and lack of experience in the interpretation of results have been the cause for their discredit. This is in contrast to the achievements in veterinary medicine in which the technique and interpretation of the agglutination test has been standardized and more or less uniformly applied. Unfortunately, this test, thus standardized, is less dependable in the case of human brucellosis. A recent discussion by McCullough (59) leads us to an analysis of the present status of this subject. The laboratory tests will be discussed in order of frequency of their use.

*Agglutination test.*—In spite of the persistent recommendation by experienced workers (57, 60) of the careful titration of agglutinins by the macroscopic method, the facility with which slide tests are performed has gained universal favor which will be very difficult to discredit. The range of sensitivity of the rapid test, wide enough to cover the needs in veterinary work, is not dependable in human brucellosis unless the reactions are of maximum intensity. It should be used, therefore, as a qualitative rather than as a quantitative test. The unquestionable usefulness of the rapid methods, and the fact that laboratory facilities are not always available, has encouraged the use of simple spot tests mostly performed with whole blood (11, 26, 35).

In regard to the quantitative estimation of agglutinins, the lack of uniformity in the results continues to be the subject of complaint (39, 60). But more important seem to be the discrepancies concerning the interpretation of well-performed titrations. It is generally accepted that during the active phase of the infection high titers of agglutination are to be expected, but the fact that in some cases titers as low as 1:80 may be found in patients with a positive culture, has resulted in some confusion concerning the proper limit of a significant reaction. In this respect the frequent observation of sudden changes in the agglutinin titer during the active phase of the infection is of interest. The titer may drop, or even become negative, to reappear in significant figures within a few days. These changes may be attributed to the presence, at a given moment, of an unusual quantity of univalent antibody (blocking effect) as shown by Griffitts in chronic brucellosis (37).

*Allergy test.*—No one questions the specificity of the intradermal test, but here again, the manner of interpretation leads to discrepancies. Also, there has been no understanding concerning the use of standardized allergens. So far, at least four different soluble substances are widely used (16, 43, 68, 73) and some still prefer diluted *B. abortus* vaccine. Furthermore, a protein (74) and two polysaccharides have been recently introduced as

allergens (3, 33). The allergy test by itself is of little diagnostic value, but its clinical significance must not be disregarded. Negative during the early stages of the infection, it follows, in a way, the various stages of the course of the disease. Reactivity may become of maximum intensity when clinical and bacteriological recovery is established. It remains for an indefinite time as an indication of past infection with *Brucella*, whether clinical or unapparent, but a skin test of unusual intensity may be the only sign of chronic infection.

*Blood culture.*—The unquestionable value of a positive blood culture has stimulated the interest of bacteriologists in developing better and simpler methods for the isolation of *Brucella*. Huddleson contributed the recommendation of tryptose, which has been very useful, although care must be taken to avoid certain lots of this substance which may contain antibrucellar substances [Schuhardt *et al.* (79)], possibly related to the cystine content of tryptose (80). Since it has been observed that brucellae are rather scarce in the blood [3 to 80 per cc. (9)], a delayed multiplication in the cultures is to be expected, for which reason transfers are recommended after five days of incubation. The use of a double medium (13), in which suspected blood is added to the broth and periodic transfers made on to the agar slant placed in the same flask, has proved to be a time and material saving device which also reduces the danger of infection to laboratory workers.

*Opsonocytophagic test.*—This test has been the subject of considerable discussion, particularly in regard to its significance in infection and immunity (Huddleson), a question which remains unsolved. As a diagnostic aid some authors (40, 41, 59) consider that when properly performed and interpreted the test is of some value.

*Leucocytic picture.*—Relatively little has been published recently on this subject. That a high percentage of the patients have a tendency to neutropenia with absolute or relative lymphocytosis, and that the altered leucocytic picture constitutes the most persistent change in individual cases, has been the common observation in the study of several thousand patients (24). In animal brucellosis, Ferguson *et al.* (32) have continued their interesting studies of the blood picture under various conditions of health and infection.

*Evaluation of the tests.*—In spite of the fact that *Brucella* has been isolated from tissues of apparently healthy individuals (63) a positive blood culture is the best proof of active infection, regardless of the absence of clinical manifestations. Comparing the other laboratory tests with blood culture it becomes apparent that a significant titer of agglutinins is next in diagnostic importance. From a group of 100 brucellosis patients (24) with positive agglutination tests, only about 80 per cent positive cultures were obtained, while from 100 cases with successful isolations of *Brucella* there may be 4 per cent negative agglutination tests. This latter discrepancy has been found with less frequency by others (57) although the differences may be due to the criterion employed in evaluating low agglutinin titers. It appears from this comparison that some cases of active brucellosis might remain undetected if only agglutination tests were used as a diagnostic criterion. On the other

hand, significant titers of cross-reactions in tuberculosis (93) and in persons who have been vaccinated against cholera (29) have been recently reported.

## Pathogenesis

An excellent summary of what is known concerning the pathogenesis of brucellosis may be found in a recent review by Meyer (65). However, the pioneering work of Smith (94) from which Huddleson reproduced in 1934 a photomicrograph of intracellular *Brucella*, and the studies of Goodpasture and his school (8a, 95), which corroborated this peculiar parasitism in infected embryos, had not been given much consideration until Meyer published his findings in a human case of *B. suis* infection (66). The periodic search by Castaneda (14) for the brucellae inoculated into laboratory animals showed that the organisms disappeared from the intercellular spaces after a few hours, only to be found within the cytoplasm of monocytes and polymorphonuclears. Several days later histiocytes, reticulo-endothelial cells, and even parenchymatous cells were seen heavily parasitized. Similar microscopic pictures could be produced by the injection of large doses of killed brucellae with the difference that parenchymatous cells were not involved (15). The usual process of phagocytosis would hardly explain the overcrowded aspect of monocytes and dust cells from the lungs, but the absence of intracellular organisms in parenchymatous cells shows the true nature of the accumulation of live brucellae within the cytoplasm of these cells. It is of interest that at least two of the known intracellular parasites, when killed, can reproduce certain microscopic aspects seen during the infection. This might help in the understanding of the relationship between the infecting agents and their hosts.

The intracellular parasitism of *Brucella* must be taken into consideration in the interpretation of the symptomatology, histopathology and particularly in the adequate treatment of the disease. The tenacity with which brucellae remain attached to their host, in spite of an active cellular and humoral immunity, depends, obviously, on their facility to continue parasitism within certain cells of the spleeen, liver, bone marrow, etc. As a consequence of the persistent stimulation, the patients become hypersensitive to *Brucella* or its metabolic products.

It is well known that certain clinical aspects of brucellosis have been confused with those of tuberculosis, that some histopathologic lesions, and even several mechanisms of allergy and immunity in the two diseases, are alike, yet the biology of the microorganisms in the two cases is entirely different. One may wonder if the chemical composition of the organisms has not a great deal to do with this rather strange similarity. We recall in this connection the acid and alcohol fastness shown by chloroform extracts (10) obtained from *Brucella*.

## Pathology

The three types of *Brucella* are capable of producing similar pathologic pictures but necropsy findings seem to indicate that differences may appear if large groups of cases are compared. The classic description of experimental

infection by Fabyan has often been corroborated in human infection and the most recent work by Spink and collaborators (84, 88) has added unquestionable evidence to these early findings. The liver seems to be the organ where granulomas are more readily found, although the spleen and other organs may also be the site of abundant lesions. The study of 11 cases of *B. abortus* infections published by these authors has shown granulomas in material obtained by liver biopsy. The small amount of tissue excised showed lesions which indicate that the frequency and number of granulomas in that organ must be quite important. These findings conflict with the early observations on Malta Fever cases which hardly mention any special histologic features that might be confused with tubercular granulomas. Mazza's study of cases of *B. melitensis* infection in Argentina (58) gives evidence that the liver is the site of important inflammatory and necrotic lesions, but he does not mention granulomas. In a study of three fatal cases of melitensis infection Nyka (69) failed to find granulomas. Later the same author (24) studied four of our melitensis cases. Three of them revealed the same negative results but in the fourth case granulomas were readily observed. With respect to the possible influence of brucellosis on the pathogenesis of cirrhosis of the liver, the findings in *B. abortus* and *B. melitensis* infections are not the same. Spink *et al.* (88) reviewed the cases of cirrhosis most likely related to *B. abortus* infection and added their own observations which suggest a causal relationship. In 14 necropsies performed at the General Hospital of Mexico City, Nika found two cases of cirrhosis of the liver (69), one of which was acquired previous to the infection. Moreover, this worker and also Costero (19) consider cirrhosis of the liver a common finding in random necropsies in Mexico.

The first histopathologic study of human lesions produced by *Brucella* was presented by Lowbeer (56). Spondylitis in cases of *B. melitensis* infection is rather frequent in Argentina (78, 91), from where necropsy material was sent to Lowbeer for histological study. This author reported that

> the process is characterized by an apparently rapid destruction of bone by granulation tissue composed of predominantly mononuclear cells containing bizarre multinucleated cells of foreign body type and showing a tendency to extensive caseous-like necrosis occurring in melitensis infection as well as suis infection. This granulation tissue becomes rapidly encapsulated by scar tissue, and extensive repair with new bone formation takes place which gives the process its self-limiting, but rather stationary character.

The fundamental lesions found in experimental as well as human brucellosis are without question a response common to infection by the genus *Brucella*, but since differences in virulence and pathogenicity have been demonstrated, one must not be surprised also to find differences in the tissue response to the three types of *Brucella*.

## Therapy

During the last two years considerable advance has been made in the therapy of human brucellosis. A summary of this subject has been presented

by Eisele (27) who, at the same time, evaluates the results so far obtained with antibiotics and warns against premature conclusions that might have a fate similar to that of the so many failures in the past.

After sulfonamides had been thoroughly tested and discarded in antibrucellar therapy, Huddleson revived the interest in these drugs (45). His experiments demonstrated that sulfadiazine increased to a considerable degree the bacteriostatic effect of the antibody-complement complex. He then applied this new concept to human therapy. Data on the results of this method are not available, except for the observation of a number of cases treated in Mexico under Huddleson's instruction (64) and of some patients studied by Eisele (27). It seems that a rapid, though transient, clinical improvement follows this treatment.

In Italy, Del Vecchio (23) claims vitamin K has a marked antibrucellar activity and some clinicians report favorable results in human brucellosis. Judging from their protocols, clinical recovery is rather slow. In evaluating their claims, one must not forget that Italian scientists are well acquainted with this disease and that in Europe at present the more active antibiotics are not available in sufficient quantities.

*Streptomycin.*—A recent review by Spink (85) has summarized the most important findings in the study of this drug. Experimental therapy in animals had given conflicting results, and several papers published in 1947 gave a rather discouraging impression of the value of streptomycin in human brucellosis. It was surprising that the concentrations of the drug in the patient's serum were high enough to have been active against *Brucella*. Pulaski & Amspacher (76) and independently Eisele & McCullough (28), however, reported that the combination of sulfadiazine with streptomycin produced better therapeutic results. The work of Spink *et al.* (87) provided adequate evidence of the usefulness of the combination, which they considered a result of synergistic action rather than the additive effect of the two drugs. A significant number of patients infected with *B. melitensis* was treated in Mexico with results which had not been obtained with previous therapeutic methods (18, 86). Unfortunately, clinical and bacteriological recurrences were frequent. This, added to the toxicity of streptomycin, was the cause of the restriction in its use. Dihydrostreptomycin, however, has renewed confidence in the combination, the synergistic action of which is worthy of further investigation.

*Aureomycin and chloromycetin.*—Among the organisms found to be affected by aureomycin, *Brucella* has a sensitivity to concentrations which are easily reached in the blood of human beings without toxic effects. This has encouraged the use of the drug in human brucellosis (7). The first important study of patients was reported in a preliminary note to the Second Inter-American Congress of Brucellosis (18), and was soon afterwards published in detail (86). Clinical recovery began in 72 to 96 hr. and blood cultures made during and soon after treatment were negative. They remained so in the majority of cases during two months of close observation. Later, a number of cases became bacteremic again and had one or more clinical

relapses. A comparative study of the streptomycin-sulfadiazine combination and aureomycin in a large group of patients (24) showed that the cases treated with aureomycin recovered more quickly and remained free longer from clinical and bacteriological recurrences. Recent work by Bryer *et al.* (8) and Braude *et al.* (4) corroborates the usefulness of aureomycin in *B. suis* and *B. abortus* infections. In regard to dosage, it has been observed that a minimal dose of 1 gm. per day for 10 days is enough to bring about clinical improvement and maintenance of negative blood cultures for several weeks. When the patients are treated by the intravenous route a total amount of 1 or 2 gm. for 5 to 7 days has been found sufficient to produce a beneficial effect on the clinical picture (17). The scarcity of aureomycin and the fact that this drug has been found to be active in small doses, together with the beneficial effect of the combination of streptomycin and sulfadiazine, has encouraged the use of a triple treatment consisting of 0.5 gm. of aureomycin and 3 gm. of sulfadiazine, both given orally, and 1 gm. of streptomycin administered intramuscularly. About 50 patients have been treated for six to eight days with excellent immediate results (24). The combined treatment with aureomycin and streptomycin in comparatively high doses has been reported by Herrell & Barker (42) with results which they considered better than those obtained by other methods.

According to *in vitro* (83) and chick embryo experiments (54), chloromycetin has a considerable antibrucellar action. When tested in human cases of brucellosis Leon *et al.* (54), Woodward *et al.* (92), and others (52) found an unquestionable beneficial effect, both in the clinical picture and on bacteremia. These findings duplicate those observed with aureomycin, including the disappointing fact that recurrences are often registered.

The pessimistic comment of Eisele is justified in principle, since we have no effective means of eradicating *Brucella* from their intracellular refuge. No one can deny, however, that streptomycin, aureomycin, and chloromycetin have fundamentally changed the prognosis for the brucellosis patient. On the other hand, the opinion of some clinicians that antibiotics alone are sufficient, disregarding the widely used biological methods, seems to be premature. We already have experience with nonbacteremic chronic patients in whom antibiotics are of little help. The fact that favorable reports concerning the usefulness of biological methods continue to appear must be taken into consideration before we can definitely conclude that they are no longer necessary (25, 38, 81, 90).

## Control and Eradication

In spite of the complex epidemiological aspects of brucellosis, the most important channels of infection are ingestion of contaminated food and direct contact with infected animals. Enforcement of pasteurization of all dairy products and increased propaganda in rural zones has been effective in the United States, Canada, and Northern Europe. The same recommendations have failed in Mexico, South America, and Mediterranean countries. A possible reason for this difference is that in the latter countries goats are the

most important reservoir of the disease. It is obvious that under such conditions, proper sanitary measures are exceedingly difficult of application. Furthermore, goats infect dairy cattle thus increasing the sources of melitensis infection. The incidence of brucellosis acquired by direct contact has increased everywhere, particularly because of cross-infection of animals with virulent brucellae (2, 49). Because of the importance of this epidemiological aspect of the infection, several attempts have been made to consider brucellosis as an occupational disease.

The economic importance of animal brucellosis has required the application of several plans to control and eradicate the disease. On the occasion of the recent Symposium on Brucellosis (20) this problem was reviewed by Gilman (36), whose discussion included an analysis of the causes of failures of previous plans and suggested remedies. He considered as one of the most important difficulties the misunderstanding between herd owners and officials in charge of the programs. This was a consequence of the failure of radical measures undertaken because of the success of similar campaigns for the eradication of tuberculosis. A better understanding of the pathogenesis and epizootology of the disease has resulted in the elaboration of plans made known recently by the Sub-Committee on Brucellosis of the National Research Council, U.S.A. (89). The actual situation in regard to the problem of control and eradication in the U.S.A. has been presented by Simms (82) from whom we quote:

> The future will bring eradication of brucellosis from our cattle herds as surely as the past has brought eradication of tick fever and tuberculosis. How far in the future this will be depends upon the energy, the perseverance, the knowledge of basic facts, and the whole hearted cooperation of all of us who are interested in the prosperity and the health of our people.

Porcine and caprine brucellosis are likely to follow the same path. As a consequence of the success in the eradication of animal brucellosis, the human disease will decrease or disappear altogether in those countries fortunate enough to afford these costly programs. We fear that caprine brucellosis will remain an unsolved problem in many countries where economic and educational conditions are the main obstacles to the eradication of the disease.

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# INFLUENCE OF NUTRITION IN EXPERIMENTAL INFECTION[1]

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*Quod aliis cibus est, aliis fuat acre venenum.* (*Lucretius. De Rerum Natura,* BK. IV, line 638)

During this post-war period, a number of excellent symposia and reviews of the broad problems of nutrition have been published, as well as many from narrower aspects. Special mention should be made of those in the broader field by McHenry & Leeson (1), Keys (2), and, in more restricted phases, those by Aycock & Lutman (3), Schneider (4), Cannon (5), and by Clark *et al.* (6).

So universal is the belief that deficiencies in diet are important factors in susceptibility to pestilence and so obvious seems the conception that one can improve his well-being and at the same time his resistance to infection by proper eating, that it is difficult to separate facts from fancies. War, famine, and pestilence have marched hand in hand through the ages, and the wars of this generation have brought bitter poverty with devastating misery and starvation to vast populations. Only a few favored areas are not tragically and intimately aware that Malthus was presenting more than an academic theory. "And the ill-favoured and lean-fleshed kine did eat up the seven well-favoured and fat kine" (7).

Restricting our attention primarily to experimental infections, we wonder whether it is possible, with our present knowledge, to determine with any exactness how marked is the effect and how definite is the result of specific dietary deficiencies and whether any threads of correlation may be observed. Because of the restrictions in space and the excellent reviews available, effort has been made to limit this survey to representative reports of the past decade. Helminth infections will not be discussed; they are well considered in the reviews already mentioned and by Culbertson (8). The field of bacterial viruses, in which some of the more fundamental observations are being made, is presented by Anderson in chapter 2 of this volume.

Although our subject is one in which environmental factors are chiefly under consideration, we must bear in mind the fundamental importance of the genetic background, not only of the hosts but also of the infective agents. Webster (9) has most effectively demonstrated that by inbreeding and selection from a common stock of mice, he could obtain two strains of animals differing markedly when infected by the gastro-intestinal route with *Salmonella enteritidis;* one strain of mice showed almost complete survival and the other

[1] This review covers approximately the period from January, 1940 to January, 1950.

a high death rate. Subsequently (10), employing the same methods, he found that resistance or susceptibility to one disease was quite independent of resistance to another, namely, mouse typhoid and St. Louis encephalitis. Four strains of mice were obtained, demonstrating, with striking differences, the four possible combinations of susceptibility to the two diseases. In the same animal he found resistance to one infection and susceptibility to the other. Furthermore, Schneider (11) found that in certain dietary experiments (to be considered more in detail later) a higher survival rate against *S. enteritidis* could be demonstrated only when a stock of heterogenetic outbred W-Swiss mice was used and when the infecting agents were also "heterogenetic."

Also, in widely differing diseases of man such as leprosy (12), rheumatic fever (13), poliomyelitis (14), diphtheria (15), and tuberculosis (16), careful analyses have shown the significance of the genetic background, both in the incidence and in the severity of the disease. Of "twin method" studies, those of Kallman & Reisner (17) in tuberculosis are among the best; in 308 complete twin families the morbidity rates show that the chance of developing tuberculosis increases in strict proportion to the degree of blood relationship. Considering the course and eventual outcome of the disease, the difference between the dizygotic and monozygotic co-twins increases to a ratio of 1:16. The influence of inheritance in resistance is well reviewed by Gowen (18).

It is generally recognized that children under admirable environmental conditions and in apparent good health are as susceptible to the common childhood diseases as are those in inferior states of nutrition and environment. Indeed, in many instances, the resistance of those from overcrowded and slum areas is greater than that of children from the "superior" environments (18a, 18b). Many unknown factors promoting this "survival of the fittest" are doubtless important. Evidence indicates, however, that much of the increased resistance is due to specific immunity following previous and repeated subclinical contacts; witness, for example, the early development of negative Schick tests in east side New York as compared with the better residential areas (19), and the early development of neutralizing antibodies for poliomyelitis virus in the serum of children in the Orient and in Mexico as compared with those in the United States (20 to 24). In short, it does not seem possible to establish at any age a direct causal relationship between discernible adequate nutrition and apparent health and natural resistance to many of the common communicable diseases.

In this review, we shall be dealing chiefly with native resistance as observed under the special conditions of laboratory studies; only rarely have experimental studies been concerned with immunity acquired through vaccination or by clinical or subclinical contacts. In all interpretations, one must be exceedingly wary in attempting to carry over to man experience with other mammals.

## PROTEINS

Of the relatively specific deficiencies, that of protein appears to have the more profound effects. The underlying foundation for our thinking in this field stems largely from the work of Whipple and his associates (25) who have

emphasized the importance of the protein stores of the liver and other tissue cells as an example of the "Wisdom of the body" (25a) and as a "reserve against adversity." With this thesis and with the fact also in mind that antibody formation is part of plasma globulin synthesis, Cannon and his colleagues (5, 26, 27, 28) have built up a convincing mass of evidence demonstrating that severe and prolonged deficiency in protein intake with the production of a hypoproteinemia causes a reduction in antibody formation and adversely influences a variety of experimental infections.

Rats and rabbits, both young and mature animals, were made markedly hypoproteinemic by prolonged feeding of diets low in protein but adequate in other respects; at times, also, the animals were subjected to plasmapheresis. Such animals responded with antibody formation against antigens such as *E. typhi* and sheep erythrocytes to a level one-fifth to one-third that of the normal controls. The deficient animals regained the capacity to produce high titer antibodies when fed diets containing adequate amounts of high quality protein or a suitable mixture of crystalline amino acids. Protein deficient rats were experimentally infected with pneumococci, Friedlaender's bacilli, or trichinae and showed definite decreased antibody production and lowered resistance as well as reduced cellular response and degree of phagocytosis. Two papers by Wissler (26) merit especial attention since they present, along with interesting correlated physiologic studies, corroborative evidence of the importance of proteins in the case of animals with active immunity acquired through vaccination. Rabbits and rats, both protein-depleted and normal, were vaccinated with formolized pneumococci Type 1 and were then challenged by intracutaneous injections of homologous virulent organisms. Antibody response, cellular reactions, and final outcome of the disease were all poorer in the depleted animals. The story is manifestly a complex one with both humoral and cellular factors involved, but the main thesis is well sustained.

Among papers from other laboratories, presenting the role of proteins in resistance to experimental infections, only a few representative ones can be cited.

Studies by the Wisconsin poliomyelitis group (29, 30) with mice maintained on different levels of protein and subsequently inoculated with Theiler's GDVII or TO virus, gave statistically negative results. Subsequently these investigators employed the essential amino acids in purified form, deleting one after another from the otherwise complete diet. With mice deficient in tryptophane, isoleucine, or valine, the clinical signs of these virus infections and also of Lansing strain poliomyelitis were markedly altered, with few animals showing signs of paralysis or definitive encephalitis and with a delayed incubation and death rate as compared with mice on optimum diets (31). In most instances, however, delayed deaths, without observed signs, complemented those with manifest virus infection; evidence indicates that most of these were virus deaths or a combination of virus and deficiency.

Especially in several tryptophane series, and with less exactness in isoleucine groups, individual titrations in mice of brain and of cord from the

deficient animals showed that the virus had multiplied with great rapidity in the brain but had not proliferated in as high titer in the cord. In the optimum controls the titers in brain and in cord were essentially equal.

Jones and his colleagues (32) with Lansing strain of poliomyelitis virus in mice found that diets low in tryptophane produced a marked delay in onset but no other modification of the disease.

Sako (33) fed groups of mice for six weeks on seven different diets varying in the proportions of protein, fat, and carbohydrate, but with minerals and vitamins adequate and the same in all of the diets. The animals were then inoculated subcutaneously with a multiple lethal dose of Type 2 pneumococcus. Those with low protein intake showed a statistically significant decrease in the post-inoculation survival time, while the animals with an exceedingly high protein intake showed a definite lengthening of the survival time.

Guggenheim & Buechler (34) in studies begun with Kliger (see VITAMIN section of this chapter) found that protein restriction of four weeks' duration reduces the bactericidal power of peritoneal fluid of rats injected intra-abdominally with *Salmonella typhimurium*. Caloric restriction had an even more marked effect.

Koerner, Getz & Long (35), working with rats that had been maintained on 15, 25, and 40 per cent protein levels for a number of generations, found that after an intravenous injection of tubercle bacilli, the animals on the 40 per cent protein diet outlived those on the lower percentages of protein by several months. In a second series, with the dosage of bacilli calculated on the basis of lung weight, the tuberculosis was less extensive, the number of organisms in stained sections smaller, the degree of localization better and the survival much longer in the rats on the 40 per cent protein diet.

In quite a different type of disease, malaria in chicks induced by *Plasmodium lophurae*, Seeler & Ott (36) found that none of the infected birds which had been kept on a high protein diet died during the observation period of 28 days. The disease was relatively benign in these birds, whereas the infection was more acute, with higher levels of circulating parasites and with a high mortality rate in the protein-deficient birds.

All recognize the large number of variables and the complexity of the pathology involved in nutrition-infection experiments, especially in animals with severe depletions; also, it is apparent that many of the variables are not studied in experiments because of the prodigious amount of labor required in following the large numbers of laboratory animals. Obviously the hope is that, by a diversity of experiments, a particular factor responsible for observed differences may be determined and that more detailed studies can then be carried out.

Of the small number of experimental studies reporting contrary results, the papers of Metcoff *et al.* (37, 38) are noteworthy for the nicety and relative completeness of their physiologic studies. They employed Sherman rats, a protein-deficient and an adequate diet, and determined the effect of these diets by infecting both groups with *S. paratyphi* and also similarly fed groups with virulent human tubercle bacilli (H37RV). In their studies they deter-

mined not only growth curves, food consumption per rat, both total amount and caloric value on the basis of 100 gm. of rat, significant bacteriologic, immunologic, and hematologic facts, but also the electrophoretic distribution of plasma proteins of all the groups, both infected and uninfected. Briefly, the authors concluded that "under the conditions of this study, dietary protein deficiency did not appear to alter the susceptibility, resistance, course, or physiologic response of the young rat to experimental tuberculosis." Also, rats infected with *S. typhimurium* appeared to respond similarly whether on the protein deficient diet or on the adequate control ration.

In examining this series, one questions whether the authors have paid sufficient attention to the recognized tenacity with which man and other mammals tend to cling to their protein stores under adverse dietary conditions. In the *typhimurium* experiments, the rats were maintained on the low protein diets only six days before infection and in the tuberculosis series on a moderately low protein diet (8 per cent casein per 100 gm.), for only a fortnight. Four weeks later approximately half of the remaining animals in each diet group were reduced acutely to a 2 per cent casein level. A study of the figures given indicates that the animals, at the time of inoculation, did not present evidences of "severe protein deprivation"; in fact they continued to gain in weight until the 2 per cent casein ration was instituted.

In the somewhat comparable paper on experimental tuberculosis by Koerner *et al.* (35) the rats had been maintained on various diets throughout their lives, in fact through a number of generations, although it must be admitted that their so-called low protein diet contained 15 per cent protein from several sources but without any casein, probably a border line level. All persons who have found significant differences in protein-depleted animals have emphasized the importance of a prolonged period of dietary restriction. This period has varied with the species and strain of animal employed, with the age and size of the animals and, of course, with the particular diet provided; with rats the period has commonly been a minimum of six to eight weeks. With adult rabbits and larger animals some investigators (25, 26) have found it desirable to hasten the hypoproteinemia by plasmapheresis, in addition to the deficient diet.

Although the periods of depletion in the Metcoff series seem unduly short to represent any actual clash with most of the other papers on experimental protein deficiency, their studies call to mind the rarity in our hospitals of cases of actual plasma protein deficiency.

Despite the increasing use of electrophoretic methods in different types of human disease (39, 40) we wish for more such studies, especially in patients with hypoproteinemia associated with nutritional anemia, but uncomplicated by infection. Bieler, Ecker & Spies (41) report that "malnourished anemic individuals at the Nutrition Clinic are remarkably free from infection despite low serum protein levels." Krebs (42) reports a single case of hypoproteinemia due apparently to uncomplicated malnutrition in which electrophoretic analysis showed low $\gamma$-globulin before, and a definite improvement in this fraction following, a high protein high calory diet. Even in slow starvation among prisoners-of-war, Leyton (43) reports that

the "liability to the common acute infections did not seem to be increased" although "if infection ensued, the rate of recovery was slower than normal." The incidence of tuberculosis was high generally, but higher among the Russians than among the British. Many studies in the war torn countries with the numberless victims of starvation and malnutrition of all sorts emphasize the overwhelming importance of sufficient calories (2, 44) and the lesser significance of specific deficiencies. Physiologic adaptation of the human body to lower levels of food intake and to amounts of essential foods below those we have been accustomed to consider necessary has apparently occurred. Manifestly more study is needed both in man and in lower mammals, with the expectation that such studies will lead to a clearer understanding of the actual relation of the "poorer" diets to infectious disease in man. Possibly adaptive bacterial syntheses occur to a considerable degree in the semi-starved (45 to 47a).

## MINERALS

The liberal quantities of the essential mineral elements, save iodine, in the foods of our western world makes us prone to forget the origin of the word, so important to us all, "salary." Hitchings & Falco (48, 49) have demonstrated a striking relation of small amounts of another element, manganese, in the diet to a peculiar susceptibility of mice to a pneumococcus infection. They found that white mice injected intraperitoneally with Type 1 pneumococci, SV-1 strain, survived 100,000 lethal doses when fed on their basal purified diet compared with animals maintained on a variety of commercial stock diets. When certain crude foodstuffs were added to the "synthetic" diet, the susceptibility of the mice was increased; extracts of these foods increased the *in vitro* rate of growth of the pneumococci. The "susceptibility" or the "pneumococcus growth" factor was especially abundant in wheat germ and was subsequently identified as manganese. "The susceptibility of the mice steadily increased with increasing manganese content of the diet, to levels as high as 0.6 mg Mn/gm of diet." Note, however, the peculiarity of this phenomenon (50) in that it can be demonstrated with only about five strains of pneumococci of 20 examined and that some strains which are markedly stimulated in growth *in vitro* by manganese do not show the effect in the mice. "Manganese deficiency in the mice seems ruled out so that the effect appears to be due to some obscure biochemical effect in the mouse."

The Wisconsin group of poliomyelitis investigators (30, 31, 51) has shown striking differences in the "resistance" of Webster Swiss mice to Theiler's GDVII virus when deficient in several of the essential minerals. Through the use of a complete synthetic diet and carefully calculated salt mixtures, they deleted one after another of the important minerals from the otherwise satisfactory ration. The animals were challenged by intracerebral injection of Theiler's GDVII virus at a time which would bring the peak of the deficiency approximately at the end of the average incubation period. No demonstrable effect on the course of the disease was obtained by varying the level of calcium, mangesium, or chlorine in the diet; a sodium deficiency resulted in some decrease in the number of paralyses; a progressively de-

creasing incidence of paralysis was observed as the amount of potassium or of phosphorus was decreased from optimum to an essentially completely deficient level. The incidence of paralysis varied from 37 per cent in the potassium deficient groups to 78 to 95 per cent in the potassium optimum groups; similar results were obtained in the phosphorus deficient series. Deaths without any definitive signs occurred, however, in all inoculated deficient groups, approximating 100 per cent, at a time when few uninoculated deficient animals had died. It would seem more conservative, therefore, to state that the deficiency in potassium and in phosphorus had markedly altered the clinical picture rather than produced a definite increase in resistance.

In a single series studied in each case, potassium deficient mice inoculated with Lansing strain murine adapted poliomyelitis virus, similarly deficient mice challenged with western equine encephalitis, and potassium-deficient rhesus monkeys infected with M.V. strain of poliomyelitis virus, showed no significant differences when compared with normally fed animals (31).

The Philadelphia Children's Hospital group (52) in a characteristically careful study of various dietary levels and combinations of phosphorus, calcium, and vitamin D have found that the susceptibility of the deficient mice to Lansing virus was greatest on the more deficient diets. This was especially true on diets deficient both in phosphorus and in calcium.

## VITAMINS

Vitamins are so important in growth and so essential in preventing certain nutritional diseases that investigators everywhere have sought persistently to find a possible causal relation between one or more vitamin deficiencies and the elusive individual differences in natural and in acquired resistance. In attempting a survey of this portion of the field, one is impressed by the maze of the literature but not by the import of the results. The conflicting statements emphasize the complexity of the problems and the difficulty in establishing critical controls. Also, we would again urge that they show the need for experimental animals with a known genetic background.

The tissue changes in certain of the vitamin deficiencies have been effectively summarized by Wolbach & Bessey (53). On this basis, as well as on clinical and experimental grounds, one realizes that many of these pathologic changes, especially if severe, provide adequate sites for secondary infections. This emphasis on secondary rather than primary infections and a stress on the nonspecific alterations in underlying physiology as a result of vitamin deficiencies can be observed in many of the recent reviews and texts.

Indeed, the excellent critical review by Aycock & Lutman (3) points out that as far as our present knowledge goes, vitamin deficiencies cannot be accepted as a general "epidemiologic principle." "The indications are that only deficiencies of certain vitamins affect susceptibility to certain types of infections and that these occur only in limited areas where these vitamin

deficiencies reach a sufficiently severe degree to produce tissue changes which are favorable sites for secondary infection."

Both Zinsser (54) and, more recently, Sigerist (55) have presented in a forceful fashion, yet with due attention to accuracy, the factors of over crowding and the revolution in living that occur among war stricken, half-starved peoples. They emphasize the secondary role played by famine and place primary emphasis for the pestilences that follow on the gross crowding, the closer association between man and the lower animals, especially rats, and the consequent interchange of pathogenic parasites or their arthropod carriers. "Cherchez les virus"—and the prepared soil.

## Vitamin A

Less stress has been placed on vitamin A as the deficient factor in the experimental infections of the last few years. Admittedly, a severe deficiency in this factor produces atrophy of the epithelium, followed by a keratinizing metaplasia. Secondary infections may follow in these sites, but Mellanby's term, anti-infective vitamin, has gradually fallen into disuse as evidence has shown its inadequate basis. In the latest considerable text in the field of nutrition, McLester (56) states that "investigation has shown the term to be misleading." Wolbach (57) states "Vitamin A can no longer be regarded as an anti-infective vitamin since the 'abscesses' described by nutritionists have proved to be glands or ducts distended with keratinized epithelial cells, the result of reparative replacement of atrophic epithelium by stratified keratinizing epithelium."

Vitamin A deficiency in rats, even when extreme, caused no appreciable loss of resistance to murine typhus (58).

Similarly, vitamin A deficient rats, with salmonella infection, showed a slight increase in susceptibility in extreme avitaminosis, but equal susceptibility was demonstrated in inanition controls when animals were given adequate vitamins but the same caloric intake as those on the vitamin-free diet (59).

## B Vitamins

In the past few years, the influence of the B vitamins in experimental infectious diseases has been actively studied. This has been due in part to their manifest importance in intracellular physiology, in part to the dramatic discovery of new members of the complex, but probably even more to the availability of pure compounds which has made possible the use of synthetic diets, thus eliminating some of the many confusing variables in nutrition studies. So varied are the results in different infections and with different deficiencies, that one can, in the space allotted, cite only representative reports indicating probable trends.

*Protozoa.*—In a number of experimental protozoan diseases one finds, contrary to usual expectation, that certain deficiencies provide a basis for increased resistance to the infection. Representative of these are the findings of Brackett, Waletsky & Baker (60) that a pantothenic acid deficiency in chickens inhibits the growth of *Plasmodium gallinaceum*. The birds receiving

pantothenate reached a usual peak of 50 per cent or more parasitized erythrocytes while the deficient chickens showed only 3 per cent or less infected cells.

Similarly (61), riboflavin seems essential to another one of the avian malarial parasites, *Plasmodium lophurae.* Chicks fed a diet low in riboflavin and subsequently injected with parasitized red cells developed infections with a peak parasite number one-fifth as high as in the birds receiving an adequate amount of the vitamin. Chicks which were fed the adequate diet, but one-half the amount so that they grew as slowly as the birds on the riboflavin deficient diet, developed heavier infections that the controls on *ad libitum* amounts of the complete diet. This would seem to rule out inanition as the significant factor and indicate that the observed results were due specifically to the riboflavin deficiency. In spite, however, of the lower parasitemia in the deficient chicks, more deaths occurred among the deficient infected birds than among either the infected nondeficient or the uninfected deficient controls. Although the riboflavin deficiency rendered the chicks unsuitable as hosts for the parasite, peculiarly it also made the chicks more susceptible to the infection.

Apparently we have in each of these deficiencies a competitive demand for the vitamin in question, on the part both of the host and of the parasiste.

A greater number, however, of the studies in experimental protozoan infections record that many vitamin B deficiencies render the animals more, rather than less susceptible. Typical of these is a report by Caldwell & György (62) who induced biotin deficiency in rats by feeding them a diet rich in egg white. These deficient animals and well fed controls were then inoculated with *Trypansosma lewisi;* the former showed much heavier and more prolonged infections than did the control animals. Significantly higher parasite counts were observed even in slightly deficient animals, thus suggesting a specific effect of the biotin deficiency. No deaths from trypansomiasis occurred in rats fed on the control diet.

Similarly, a biotin deficiency adversely influences experimental avian malaria. Trager (63) found that moderately deficient chickens or ducks showed a parasitemia about twice as heavy as in the control non-deficient birds. This was true for *Plasmodium lophurae* both in chickens and in ducks and for *P. cathemerium* in ducks. Specificity was suggested by the fact that a very mild degree of biotin deficiency sufficed to increase susceptibility and on the other hand a marked degree of pantothenic acid deficiency had no effect. Confirming observations were made by Seeler, Ott & Gundel (64) with *P. lophurae* in deficient chicks.

A deficiency in "folic acid" (65) and, as previously stated in this review, in protein (36) tends to decrease the resistance of chickens to *P. lophurae,* whereas riboflavin deficiency reduces the severity of this infection (61).

*Bacterial infections.*—In studies by Wooley & Sebrell on pneumococcal infections in mice (66) excellent procedures were followed: use of purified diets, deficiencies manifest at time of inoculation, a natural route of inoculation (intranasal insufflation), use of blood-broth as a control inoculation mass, a known stock of mice, uninoculated deficient control groups, etc. By

using only minimal amounts of riboflavin or thiamine with their otherwise standard diets, followed, after a deficiency had been established, by intranasal insufflation with pneumococci (Type 1) they found that more mice died in the inoculated deficient groups than in the inoculated normally fed controls. Inanition as the possible responsible factor was checked in part by a paired litter mate feeding experiment in the riboflavin series but not in the thiamine series. The death rates were quite variable and in some series definitely low so that the figures are less significant. Peculiarly, also, "the daily administration of riboflavin or thiamine in amounts 5 to 10 times that in the control diet, to the mice on diets deficient in these substances, respectively, at the time of the inoculation with pneumococcus Type 1, did not reduce the number of animals dying from the infection." One is left, therefore, in doubt as to the factors involved in their observations.

Other B vitamin deficiency studies with pneumococci as the invading organism make this question even more pertinent. Day & McClung (67) report no significant differences in susceptibility of rats or mice, deficient in pantothenic acid, to intraperitoneal injection of Type 1 pneumococci. Robinson & Siegel (68) using Type 1 pneumococci by an intratracheal route through an incision in the neck, also found no differences in the rats deficient in riboflavin or pantothenic acid, as compared with rats receiving the same basal diet with adequate quantities of the vitamin under study. Their figures present a slightly unfavorable, but possibly not significant, difference in the thiamine or pyridoxine deficient rats. Inanition had no apparent influence on the resistance. Interesting, however, and reminiscent of the work of Hitchings & Falco (49) is their observation that the animals on a stock diet *ad libitum* were more susceptible than those on experimental diets.

Kligler and his colleagues (69) have continued their careful studies (some already cited under vitamin A) employing both rats and mice with peroral introduction of *S. typhimurium* and in the case of some of the mouse experiments, spontaneous outbreaks of salmonella infections. Contrary to their studies with vitamin A, they report increased susceptibility as compared with controls in the riboflavin deficient mice during a spontaneous salmonella outbreak. Fatalities were three times more frequent in the deficient group and positive cultures from liver and spleen three to four times more frequent than in the controls. The food intake was almost normal through the significant weeks of the experiment, so inanition controls were not considered necessary. Isocaloric paired-fed controls given a full vitamin ration showed an intermediate degree of susceptibility between the riboflavin deficient and the controls.

In their thiamine deficiency series (70), an increased susceptibility in the deficient rats proved to be due largely to inanition. With mice, however, paired feeding series suggested that the lowered resistance was due to the thiamine deficiency. Their biotin deficient series (71) gave the most convincing results of a specific relation between the deficiency and lowered resistance both in mice and in rats.

The relatively mild infections in the rats, the very considerable dependence on cultures rather than fatalities, and the use of rice flour-yeast

mixtures rather than more highly purified diets, makes it difficult to use the term "unequivocal" which the authors employ.

*Rickettsial and virus diseases.*—Experiments with these more completely obligatory parasites offer interesting contrasts, in that we find no instance in which dietary deficiency has increased resistance to rickettsial infection, whereas several deficiencies appear to cause increased resistance to some experimental viral diseases. What seems to be a generalization in this regard was presented some years ago by Zinsser and his co-workers (72) who found that rickettsial growth was most active "when the tissue has ceased respiring, and is either not viable at all, or has lost much, possibly all, of its metabolic acitivity," whereas a typical virus, equine encephalitis, multiplied most actively during the first two days of incubation when tissue respiration was most active.

This conception has been confirmed and amplified, especially for the rickettsiae, by several investigators. Cox (73) found that more active multiplication of rickettsiae takes place in the yolk sac between 34°C. and 37.5°C. and very little growth at 40°C. Pinkerton and his colleagues (74) reported that starvation diets increase the degree of infection to murine typhus both in rats and in guinea pigs. They have also reported a striking and apparently specific loss of resistance to murine typhus in rats that had been fed rations deficient in riboflavin. This lack of resistance occurs even in early stages of the deficiency; the administration of riboflavin to moribund rats teeming with rickettsiae in all organs causes complete recovery within twenty-four hours. They suggested that since riboflavin forms an essential component of an important respiratory enzyme, the lack of resistance in the riboflavin deficient animals might be a direct result of lowered intracellular respiration,

Greiff & Pinkerton (75) found in further studies that when potassium cyanide was injected into the yolk sac in amounts lethal for the embryo, that rickettsiae multiplied freely at 40°C. *p*-Aminobenzoic acid which, as is well known, is strikingly rickettsiostatic, increased the oxygen consumption in eggs to a degree of about 50 per cent.

Under many experimental conditions, therefore, rickettsial growth seems to be inversely proportional to cellular respiration.

In experimental viral infections, on the other hand, many observers (6) have demonstrated that a variety of intercurrent illnesses or severe dietary deficiencies either increase resistance or markedly mask the usual signs of the disease. Many studies have been carried out with virus diseases of the central nervous system and with a selected strain of albino mouse. (We have already reviewed the effects of some mineral deficiencies.)

Of the B vitamins, lack of thiamine has produced the more significant results. A thiamine deficiency in mice injected intracerebrally with Lansing strain poliomyelitis prolongs the incubation period and reduces the incidence of paralysis as well as the mortality rate, as compared with control groups on an unrestricted complete diet. The anorexia of thiamine deficiency is in part responsible for the observed differences, but paired litter mate inanition studies indicate some specific relation. With Theiler's GDVII virus, similar alterations are induced (30, 52a).

Among the more searching virus studies, those of Sabin, Olitsky and their colleagues (76, 77) have demonstrated a dietary basis for some of the observed differences in susceptibility of young and adult mice to the viruses of vesicular stomatitis and equine encephalitis. Several peripheral routes of infection such as nose, peritoneal cavity, skin, or muscle are readily effective to essentially 100 per cent in young mice and almost completely ineffective in adult animals. During maturation, barriers develop which are apparently unrelated to humoral immunity or to those gonadotropic and pituitary hormones tested, and which effectively block the routes of invasion to the central nervous system that are readily available in young animals. By withholding specific factors such as thiamine or riboflavin and presumably other heat labile B vitamins from the mothers during lactation, or similarly by weaning the animals prematurely at 2 weeks of age and continuing them on inadequate diets, the usual normal resistance to peripheral injections was delayed or prevented.

With regard to the nature of the normally occurring barriers in the older animals, the evidence suggests that the tissues may not support growth of the virus sufficiently to permit invasion of the more susceptible tissues necessary to give rise to typical signs of the infection. Subsequent studies by Sabin & Duffy (78) on the behavior of intramuscularly injected vesicular stomatitis virus in rats of different ages, indicated that failure of the virus to progress from the spinal cord to the remainder of the central nervous system in older animals was correlated with a markedly lower level of multiplication in the cord.

## UNDETERMINED FACTORS

Schneider & Webster (10, 11, 11a), in studying the natural resistance of W. Swiss mice employed a variety of cereals, singly, in combination with whole dried milk. Of these, the diet comprising 66 per cent whole wheat, 33 per cent dried milk and 1 per cent sodium chloride gave the best results in growth, fertility, fecundity, ability to rear young, and in growth of the progeny. This became their standard reference diet.

In carrying out infection tests using *S. enteritidis* introduced by stomach tube, they found that this natural whole wheat and milk diet resulted in a larger number of survivors than did a synthetic diet including all the then known vitamins. (Biotin and folic acid also were apparently adequate in the artificial diet.) This difference in survival was demonstrable only when heterogenetic outbred mice were employed and when the culture of the infective organism was also heterogeneous as to virulence. Further investigation showed that the salmonella survivorship factor is present in the wheat germ; more recently Schneider (79) has found that this factor is a precursor of the physiologically active material and that it is highly stable in the natural grain, whether wet or dry, and in acid or alkali. The chemical nature of this unknown factor has yet to be determined.

In the course of their important evolving studies in tuberculosis, Dubos and his colleagues (80) have shown marked differences in the susceptibility of three strains of mice to experimental tuberculosis on the basis of the rations consumed. The following diets, in the order given, provide a basis in nutrition

for increasing susceptibility to the injection of weighed amounts of virulent human type tubercle bacilli (H37RV).

(*a*) 66 per cent whole wheat, 33 per cent dried milk, 1 per cent sodium chloride (with or without 10 per cent gelatin).
(*b*) Fox chow pellets.
(*c*) Mixture of white bread and whole milk.
(*d*) Mixture of 85 per cent cornmeal, 5 per cent butter, 10 per cent gelatin and salts.

Supplementation of the fourth ration with the known missing vitamins did not improve the resistance of the mice. The authors suggest that the observed effects are probably due "not to a well-defined deficiency but rather to a nonspecific physiological disturbance." Again therefore, we have an unknown factor, possibly in the wheat, which markedly alters the natural resistance of mice to experimental tuberculosis.

Recently, Sengupta & Howie (81) have reported a significantly greater resistance to tuberculosis in mice reared on a complex diet, including a variety of natural grains, fish and meat proteins, bone meal, dried skimmed milk, yeast, etc., as compared with the above-mentioned diet used successfully both by Dubos and by Schneider. Many factors both known and unknown are present in their more effective diet; the report seems especially important because the authors use as their basis for comparison an excellent diet which provides factors for resistance.

## SUMMARY

In surveying the investigations which seek for possible causal relations between nutrition and resistance, a few generalizations or at least suggestive correlations appear, although somewhat dimly. One of the commonest omissions in the experimental plan is adequate control of inanition. Anorexia and reduced intake are common in so many dietary depletions that a nonspecific physiological disturbance may be and frequently is taken for a specific effect. Especially in a number of virus infections, deficiencies or intercurrent infections increase resistance or markedly retard or alter the appearance of any typical signs (6). Along with decreased food intake, both in man and in experimental animals, a voluntary dehydration occurs even though water is always available. We have been impressed with the dehydration of our mice on many deficiency diets. The loss of the sodium ion through sweating in animals with sweat glands and through urine and feces is highly important (82). Sprunt (83, 84) and also Olitsky & Schlesinger (85) have emphasized hydration and dehydration as factors in the spread and the localization of virus particles.

The importance of calories has been reemphasized by the studies among the half starved of the warring countries; experimental studies also stress this point. The possibility of adaptation to a lower intake and a beneficial alteration of the bacterial flora for the synthesis of missing vitamins has been shown both for experimental animals and for man (45, 46, 47); further studies are needed.

The essential importance of proteins (or the appropriate amino acids) for the development of antibodies and effective phagocytosis of invading parasites has been demonstrated, but depletion must commonly be severe for such demonstration. Many alterations of diet will change the picture of certain virus diseases to which mice are susceptible. Frequently this appears to be due to failure of the virus to grow vigorously. If the "resistant animals" are subsequently placed on a complete diet, the typical clinical picture develops in a high percentage. Of the various B vitamins studied by different investigators, biotin deficiency has given the most consistent results in several diseases; the wide distribution of this vitamin makes it unlikely that this difficulty occurs in nature.

Interference phenomena, well reviewed for viral infections by Syverton (86) and philosophically discussed by Woolley (87, 88) from the point of view of analogues, offer an explanation for many of the observations and represent one of the more important lines for future study. The generalization by Zinsser (72) some years ago that rickettsiae grow better as the activity of host cells is lowered, while viruses require fully active cells, has been in part confirmed.

Of great interest is the heightened resistance to salmonella and to tuberculous infections through the use of diets containing unknown factors apparently present in wheat germ and/or milk. These unknown factors are especially significant in that the experimental evidence supports clinical findings that diet is a factor in resistance of man to tuberculosis. Also, the evidence gives us renewed confidence in adequately chosen experimental models.

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